EVALUATION OF CLINICO MYCOLOGICAL ASPECTS OF ONYCHOMYCOSIS

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DECLARATION

I, Dr. N. Subathra solemnly declare that the dissertation entitled **"EVALUATION OF CLINICO MYCOLOGICAL ASPECTS OF ONYCHOMYCOSIS"** was done by me at Coimbatore Medical College Hospital during the period from April 2010 – June 2011 under the guidance and supervision of **Dr. V. Sadhiqua, M.D., DGO,** Associate professor of Microbiology, Coimbatore Medical College, Coimbatore.

This dissertation is submitted to the Tamilnadu Dr. M.G.R. Medical University towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch – IV) in Microbiology.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

Place:

Date: **Dr.N.Subathra**

CERTIFICATE

This is to certify that the enclosed work **"EVALUATION OF CLINICO MYCOLOGICAL ASPECTS OF ONYCHOMYCOSIS"** submitted by Dr.N.Subathra to the Tamilnadu Dr. M.G.R. Medical University is based on bonafide cases studied and analyzed by the candidate in the Department of Microbiology, Coimbatore Medical College Hospital during the period from April 2010 – June 2011 under the guidance and supervision of **Dr. V. Sadhiqua, M.D., DGO,** Associate professor of Microbiology and the conclusions reached in this study are her own.

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CONTENTS

ABSTRACT

Background and Objectives:

Onychomycosis, a fungal infection of nail and are caused by dermatophytes, non dermatophytic moulds and the yeasts. It showed varied mycological and clinico epidemiological pattern in various part of the country. This study was carried out to document the mycological and clinico epidemiological pattern of onychomycosis in Coimbatore (South India), to analyse the sensitivity of mycological techniques (Microscopy and culture) and to carry out the anti fungal susceptibility testing of the isolates.

Materials and methods:

Eighty five clinically diagnosed cases of onychomycosis attending the Dermatology Out patient Department of Coimbatore Medical College Hospital over a period of 15 months from April 2010 – June 2011 were taken for this study. Direct microscopy (40%KOH) and the fungal culture were done. Anti fungal susceptibility testing of the isolates were carried out by CLSI Broth microdilution method (M38 A & M27 A2).

Results:

These 85 patients were between 6 – 65years of age. Men were commonly affected than Women with a ratio of 2.7:1. Distal and lateral subungual onychomycosis was the commonest clinical type (56.50%). Finger nails were commonly affected than toe nails (3.5:1).Majority of patients were farmers, office workers and housewives. The culture and microscopy positivity were recorded in 54.12% and 50.59% of cases respectively. Dermatophytes and Non dermatophytic moulds (NDM) were isolated in 28.2% and 15.3% of cultured nail samples while the Yeasts were isolated in 10.6% of nail samples. The

dermatophytes were T.mentagrophyte (16.5%) and T.rubrum (11.8%). The commonest NDM was Penicillium species (10.6%) while other NDM were A.niger (2.4%), A.flavuus (1.2%) and Cladosporium (1.2%). Tenia corporis (8.7%) was the skin infection significantly associated with onychomycosis. In vitro susceptibility data of the isolates showed that Terbinafine has got the lowest MIC (≤ 0.03μg/ml) for all the filamentous fungi and fluconazole has got the lowest MIC (2 μg/ml) for the Yeast isolates.

Conclusion:

Onychomycosis is a signifigant health problem and shows varied clinico mycological pattern in this area. The role of NDM as the causative agents in onychomycosis is significant hence the accurate mycological diagnosis and appropriate treatment.

Key words: Onychomycosis, Clinico – mycological pattern, invitro susceptibility.

INTRODUCTION

Onychomycosis is a term used to describe fungal infection of nails caused by three groups of fungi namely dermatophytes, yeasts and non dermatophytic moulds.

The word onychomycosis is derived from Greek - 'onyx', nail and 'mykes', fungus.[3, 31]

Onychomycosis affects 5% of the population worldwide representing 20-40% of onychopathies and 30% of the mycotic cutaneous infections [3, 4, 5] The worldwide incidence of onychomycosis is increasing and a number of factors such as age, sex, occupation, climate, living condition, etc contributing to this rise. Clinical and mycological features of onychomycosis show variation with time and place.[4, 5, 7]

Previously regarded as contaminants, the yeasts and moulds are now increasingly recognized as pathogens especially in immuno compromised individuals like patients with diabetes, HIV/AIDS, steroid therapy and cancer chemotherapy[20]

In onychomycosis, the infected nails serve as a chronic reservoir of infection which can give rise to repeated mycotic infection of skin, causing considerable disfigurement and lysis of nails leading to increased morbidity. The nail changes in onychomycosis mimic other nail disorders, such as psoriasis, chronic onycholysis, lichen planus, nail trauma, etc. So it is of significance to suspect onychomycosis, perform mycological diagnosis and undertake appropriate treatment.[17]

The fungal infections of nail do not respond well to treatment, the management of onychomycosis continues to be problematic despite the advent of newer antifungal agents. The duration of treatment is also very long which may lead to potential side effects, poor compliance by the patients and as a consequence, emergence of drug resistance.[21, 30, and 47]

Successful treatment depends on the ability of the given antifungal to eradicate the fungal isolate. In order to predict this ability, in vitro susceptibility testing becomes helpful because it can help the clinicians to choose the appropriate drug for the treatment.

So it is advisable to obtain a definitive diagnosis by standard mycological techniques, to determine the various clinical patterns and to select appropriate antifungal drug for the treatment of onychomycosis by antifungal susceptibility testing, hence the study.

AIM AND OBJECTIVES

OVERALL AIM: To evaluate the mycological and clinico - epidemiological aspects of onychomycosis in Coimbatore District.

Specific Objectives:

1. To isolate and identify the fungi from the clinically diagnosed cases of onychomycosis by standard mycological techniques.
2. To study the predominant fungi causing onychomycosis and their varied clinico – epidemiological presentation in Coimbatore.
3. To determine the MIC of anti fungal drugs for the filamentous fungi and yeast isolates by CLSI M-38 A & CLSI M-27 A2 Broth micro dilution Method.
4. To do the comparative evaluation of Microscopy and Culture in the laboratory diagnosis of onychomycosis.

REVIEW OF LITERATURE

HISTORY

Mycology, the study of fungi which came into existence when Hooks in 1677 studied the yellow spots on the leaves of Desmark rose and found the filamentous organism with the help of magnifying lens. Subsequent studies were carried by Malphigi (1686), Mitchelli (1729) and Linnaeus (1752)[1]

In 1835, Augustino Bassi was the first to elucidate the microbial nature of deadly disease of silkworms (Bombyx mori) and that a mould now known as Beauveria bassiana was the cause of disease of the commercially available silkworm. This was the first organism to be recognized evoking a disease.[1]

There have been few studies of incidence of onychomycosis in the general population (Grimmer et al, 1954; Langer et al, 1957).[2]

In 1904, Dubendorfer described the candidal onychomycosis. Until late 1990s Onychomycosis was a poorly discussed topic in medical science. Even in developed countries, this infection has been high lighted only in the last decade.

Cases of onychomycosis represent 30% of diagnosed superficial fungal infections and are caused by three groups of fungi: dermatophytes, yeasts, and nondermatophytic moulds (NDM). The majority of toenail infections are caused by dermatophytes; Trichophyton rubrum is isolated with the greatest frequency. NDM represent 3% of the nail infections in a temperate country such as the United Kingdom but a much higher proportion in tropical countries.

STUDIES ON ONYCHOMYCOSIS:

In October 1987 J. Andre, M.D., G. Achten, M.D., at University of Brussels, Belgium conducted a study on Onychomycosis showed that the dermatophytes are mostly responsible for onychomycosis. They isolated two anthrophophilic dermatophytes, T.rubrum, T.mentagrophyte var interdigitale with four times higher the incidence of the first pathogen[8]

In the largest series of 79 patients with SWO from Italy, in 1994 the most common pathogen was Trichophyton interdigitale (73%), followed by Fusarium species (11%), Aspergillus species (6%),Trichophyton rubrum (5%), and Acremonium species (3%). These percentages vary in different studies, but T.mentagrophyte var interdigitale is constantly highest on the list.[11, 12, 13]

Candida albicans was the predominant isolate in two studies (1999, RSN Brilhante et al at Northeast Brazil and M Gerami shoar et al 2001 at Tehran).In their studies finger nails were the most frequent anatomic site. Finger nails were affected more in women; where as predominant site involved in males were toe nails.[10]

INDIAN SCENARIO:

Various Indian workers have reported the incidence to be at 0.5 – 5% in the general Population[9]

In 1979 Prasad and Prakash[6] conducted a study on onychomycosis at Ranchi, India. It showed the prevalence rate of 10.2%

In Jan2000, a five year study by Kaur et al in among 400 patients of clinically suspected cases of nail infections at dermatology out patient department in New Delhi showed the presence of onychomycosis in 218 cases (54.5%) by culture and/or direct

microscopy. Finger nails were the most frequent anatomical site (65%). Regarding filamentous non dermatophytic moulds, Aspergillus species, Fusarium species, Alternaria species and the Penicillium species were the most frequently isolated. Occasionally Bipolaris, Nigrospora, Curvularia, Scedosporium apiaspermum were isolated. The disease was most prevalent in the 25 -45 age group and the ratio of male to female patients was approximately 1:1.[7]

In 2003, Grover conducted a study of onychomycosis in 50 patients at Air Force Hospital, Bangalore in which Dermatophytes grown in 14 (23.7%) cultures, NDM in 13 (22%), candida in 10 (16.8%), no growth in 22 (37.2%). Commonest species among dermatophyte was T.rubrum. Among NDM Aspergillus spp was most common isolate.[22]

In North India a three year study in April 2004 by Smita Sarma, MD, showed the most common fungal isolates were dermatophytes (49.5%), followed by Candida spp (40.4%). Of the dermatophytes, Trichophyton rubrum (47%) was the most common isolate, followed by Trichophyton tonsurans (20.4%). Of the Candida spp., Candida albicans was the most common (60%). Distal and lateral subungual onychomycosis was the most common clinical pattern (62%), followed by total dystrophic onychomycosis (20.2%).[15]

In January 2005, Veer et al conducted a study at Skin and VD, OP department of Government Medical College and Hospital, Aurangabad from 88 suspected cases of onychomycosis. Direct microscopy was positive in 72 (81.8%) and culture was positive in 43 (48.8%) cases. Culture results showed growth of dermatophytes in 26 (29.5%) cases, NDM in 12 (13.6%) and Candida spp. In 5 (5.6%) while 45 (51.1%) yielded no growth. Among dermatophytes, T.rubrum was the commonest etiological agent followed by T.mentagrophytes. Among NDM, Aspergillus spp was the most prevalent followed by Alternaria spp, Curvularia spp, and Fusarium spp. Commonest age group affected was above 31 years. Male to female ratio being 1.8 :1. Finger nails were more frequently than toe nails

with the ratio of 3:1. DLSO was the more common clinical pattern (50%) followed by PSO (20.4%), SWO (2%), TDO (14%0 and paronychia (10.2%).[17]

Gupta M et al, in 2006, conducted a study of 130 patients from OP department of Dermatology , Venerology and Leprosy at Indra Gandhi Medical College , Shimla, Himachal Pradesh, India showed that the prevalence of onychomycosis higher among farmers and office workers (20% each). Fingernails were commonly involved (56.9%). DLSO was the most common clinical type (73.1%). Dermatophytes isolated were T.rubrum (32.6%), T.mentagrophytes (6.1%0 and T.verrucosum (2.1%). Aspergillus spp was the most commonly isolated NDM (6.1%) while other detected mold were Acremonium spp, Fusarium spp, Scopulariopsis spp, Curvularia spp and Penicillium marneffei. [19]

In 2007, a two year study by Adhikari et al from 34 clinically suspected cases of onychomycosis at Gangtok, Sikkim showed that trichophyton tonsurans (44.44%) was the commonest isolate followed by T. mentagrophytes (22.22%), T.rubrum (11.11%), T.verrucosum (11.11%) and M.audouinii (11.11%). Most common NDM isolated was Aspergillus niger (21.43%) followed by Penicillium marneffei (14.28). Direct microscopy for fungal elements was positive in 32 (94.12%) cases and 28 (82.35%) cases were positive for culture. Commonest age group affected was 21 – 30yrs. Male to female ratio was 1.125:1.[20]

Kaur et al in 2008 conducted a study at Maulana Azad Medical College, New Delhi from 60 patints of clinically suspected cases of onychomycosis. The study showed tthat T.rubrum was the commonest isolate (46.67%). T.mentagrophytes and Candida albicans accounted for 20% and 15.56% isolates, respectively. Isolation rate was higher by drilling compared to scraping, the rates being 83.33% and 66.67%, respectively. The highest incidence was found in the age groups between 21 – 30 years. The disease was more common in males. Finger nails were most commonly involved than toe nails.[18]

In 2008, a study by Das et al in Kolkata from 85 cases of onychomycosis showed the culture growth of fungus in 44 (51.76%) cases. Predominant agents were dermatophytes - T.rubrum (29.54%) species being most common. Aspergillus niger (18.18%) among NDM was the most common isolate. Candida albicans (11.78%) was the predominant yeast[21.] Positive results were more with culture (94.45%) than with KOH preparation (63.64%) [21]

ROLE OF NON DERMATOPHYTIC MOULDS AND YEASTS IN ONYCHOMYCOSIS:

With the increasing number of immuno compromised patients, many species of fungi originally regarded as laboratory contaminants are now considered to be agent of onychomycosis and may sometimes also affect immuno competent patients. (Guilhermetti et al).[25]

Malasaaesia furfur has also been reported as one of the NDM causing onychomycosis. (Silva V et al, Turkey, 1997)

Gibas et al in 2002, identified Onychocola kanei sp. nov., by DNA analysis at University of Toranto, Canada.[27]

In 2005, Pontarell et al, reported Sytalidium dimidiatum from two cases of onychomycosis in Santa Catarina, Brazil[29]

In a study of 250 HIV infected patients at Mumbai by Surjushe A et al in 2007 showed microscopy postitivity in 49 cases, culture growth in 32cases. Most common clinical type – TDO i.e. in 33 (55%) cases, followed by DLSO in 21 (35%) cases, PSO in 5 (8.33%) cases, SWO in one case. Commonest agents isolated were NDM, in 19 (31.66%) patients followed by dermatophytes in 13 (21.66%) patients. Of the NDM, Aspergillus nigar was the commonest isolate and other NDM agents isolated were Cladosporium spp., Sctalidium hyalinum, Penicillium spp., and Gymnoascus dankaliensis. [24]

In 2008, Hafidh reported Cladosporium spp., as the causative agent of white superficial onychomycosis in University of Bagdad, Iraq. Other non dermatophytic molds identified in his study of toe nail onychomycosis were Alternaria spp., Aspergillus spp.[28]

A new species, Aspergillus persii was isolated as the causative agent of onychomycosis by Zotti et al in 2009 at University Genoa, Italy. [26]

Summerbell et al suggested that non dermatophytic moulds and yeasts identified in nail tissue may be considered as one of the following:

1. Contaminant (species gowing in culture from dorman pars of nails)
2. Normal mammalian surface commensal organism
3. Transient saprophytic coloniser
4. Persistent secondary coloniser (coloniser of material infected by dermatophyte but incapable of remaining after the dermatophyte is eliminated)
5. Successional invader (species that can cause infection after gaining entry in to a nail via the disruption caused by primary pathogen)
6. Primary invader (able to infect and cause onychomycosis in a previously uncolonised nails)

1. Definitive diagnosis of non dermatophytic invasion of nails can be done by

"WALSE AND ENGLISH" (1966, UK) criterion [2, 55]

- Microscopy positive for fungal elements

And

- Culture also positive for NDM or yeast with the absence of concomitant dermatophytic growth with atleast five inocula give rise to same growth from same specimen inoculated at different places on culture media.

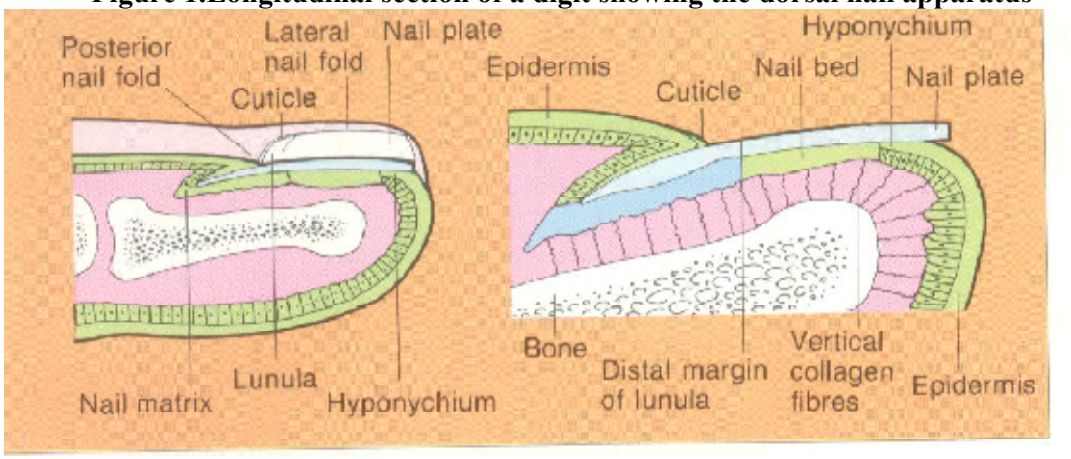
2. It has later been modified by **Summerbell et al in 2002**.[55] According to this criterion,

- Microscopy positive for fungal elements
  And
- Repeated isolation of the same fungus from the same specimen on successive two or more occasions at an interval of one week

NATOMY OF NAIL

The nail unit includes the nail plate, matrix, nail bed, proximal & lateral folds, hyponychium, and underlying distal phalanx. The components of the nail apparatus in shown diagrammatically in **figure1**

Figure 1.Longitudinal section of a digit showing the dorsal nail apparatus



Nail plate:

It is flat, roughly rectangular, slightly convex horny plate, having two lateral, one distal and one proximal edge.

Lateral nail folds:

They are the cutaneous folded structures providing the lateral borders to the nails.

Proximal nail fold:

This is cutaneous folded structure providing the visible proximal border of the nail, continuous with the cuticle.

Cuticle:

It is the horny layer extending from the dorsal and ventral aspect of the proximal nail fold and adhering to the dorsal aspect of the proximal nail fold.

Lunula:

It is the convex margin of the distal matrix seen through the nail. It is paler than adjacent nail bed. It is most commonly visible on the thumbs and great toes. It may be concealed by the proximal nail fold.

Nail bed:

The vascular bed upon which the nail rests extending from the lunula to the hyponychium. This is the major territory seen through the nail plate. The nail plate is firmly bonded to the nail bed, less so proximal to the lunula margin.

Hyponychium: The cutaneous margin underlying free nail. In this region the separation of the nail plate and nail bed begin

MYCOLOGY

Onychomycosis was traditionally referred to non dermatomatophytic fungal infection of nail but now used as general term to denote all fungal infections of nails. Tinea unguium specifically describes dermatophyte invasion of nail.

Classification of causative agents involved in onychomycosis[15, 31]

A. DERMATOPHYTES

Trichophyton rubrum

Trichophyton mentagrophyte

Epidermophyton floccosum

Trichophyton tonsurans

B. NON DERMATOPHYTIC MOULDS

Scopulariopsis brevicaulis

Acremonium spp.,

Aspergillus flavus and A. fumigatus

Fusarium oxysporum

Scytalidium dimidiatum

Scytalidium hyalinum

Onychocola Canadensis

Geotricum candidum

Culvularia lunata

Alternaria species

Penicillium species

C.YEAST LIKE FUNGUS:

Candida albicans

Microscopic and macroscopic features of each species: [31, 32]

DERMATOPHYTES:

These group of fungi are characterized by smooth thin walled pencil shaped macroconidia. Microconidia are spherical or club shaped.

1. Trichophyton mentagrophytes:

Macroscopic features:

The growth is rapid forming creamy tan to pink surface, with white, powdery to granular consistency. Reverse is buff to red or reddish brown.

Microscopic features:

Smooth walled pencil shaped macroconidia. Abundant spherical microconidia arranged in grape like clusters.

2. Trichophyton rubrum:

Macroscopic features:

A granular or fluffy white colony with a pink periphery and deep wine red reverse forms in two weeks.

Microscopic features:

Numerous or few smooth walled pencil shaped macroconidia. Numerous club shaped microconidia are borne singly along the sides of the hyphae.

B. NON DERMATOPHYTIC MOULDS:

1. Penicillium species:

Macroscopic / colony morphology:

Colonies are granular and generally with various shades of green, although yellow-brown colonies may be seen. Wine red pigment diffusing into the agar is seen in case of Penicillium marneffei.

Microscopic features:

The **penicillous or brush shaped conidiophore** is produced and it's branched into metulae, phialides from the tips of which chains of conidia are produced, tips of phialides are blunt. Conidia are spherical and regular in size and acrepetal type.

2. Aspergillus niger:

Macroscopic / colony morphology:

Surface of the colony is covered with dense **"jet black"** conidia, giving characteristic pepper effect.

Microscopic features:

Dense aggregates of single and short chains of jet black conidia that usually cover the entire vesicle. They are biseriate. (Double row of phialides).

3. Aspergillus fumigatus:

Macroscopic / colony morphology:

The colonies have shades of blue grey, green-grey, green- brown pigmentation

Microscopic features:

Single row of phialides over the top half of the vesicles. Conidia are arranged in long chains with bending towards central axis.

4. Aspergillus flavus :

Macroscopic / colony morphology:

Colonies are granular, yellow, yellow green or yellow brown.

Microscopic features:

Daisy petal like arrangement of chains of conidia with single or double row of phialides.

C. YEAST LIKE FUNGI:

Candida albicans:

Macroscopic / colony morphology: Creamy, smooth colonies.

Microscopic features:

Gram stain **:** Gram positive budding yeast cells.

EPIDEMIOLOGY:

The prevalence of onychomycosis ranges from 2 – 3% to 13% in the Western Countries in contrast to South East Asia whree the prevalence is very low. In India, the

prevalence rate varies from 0.5 to 12.5% from different studies in different part of the country.[30]

The prevalence rate is determined by age, predisposing factor, social class, occupation, climate, etc. Several studies have shown that the prevalence increases with age, reasons for which may include poor peripheral circulation, diabetes, repeated nail trauma, sub optimal immume system, poor self care. The disease prevalence is higher in HIV patients.[7]

In one study in New Delhi, India revealed that the prevalence was confirmed in 45% of the analysed patients.[7]

HOST FACTORS:

Number of host factors like diabetes, Immuno compromised states like patients with AIDS, on immuno suppressive therapy, cancer chemotherapy, and steroid use all have contribution to onychomycosis particularly by non dermatophytic fungi.[7]

Because of the above mentioned factors, non dermatophytic moulds and yeasts once regarded as contaminants are now increasingly reported as pathogens in finger nail and toe nail onychomycosis.[5]

Onychomycosis in adults is mainly by trauma due to increased out door activities, those wearing occlusive foot wear especially in business peoples and executives, office workers.

Onychomycosis is more common in males from most of the studies in various parts of the world.[17, 19, 20]Candidal onychomycosis is more common in females (house wives, cooks) which may be attributed to the frequent immersion of hand in water.[35]

ENVIRONMENTAL FACTORS:

Living environment and climate have great impact on onychomycosis.[5]The disease is lower in tropical countries (3.8%) than in subtropical countries and countries in the temperate zone (18%).[33]

CLINICAL FEATURES

Onychomycosis, the fungal infection of nail affects all age group, both sexes. It affects toenails as well as fingernails. Toe nail onychomycosis is more common than fingernails except few cases.

CLINICAL TYPES:

1. DISTAL SUBUNGUAL ONYCHOMYCOSIS (DLSO):

It is the most common type of clinical presentation in which there is invasion of nail bed and underside of the nailplate. It has two consequences – onycholysis and subungual hyperkeratosis. The dermatophytes are the predominant causative agents.

2. PROXIMAL SUBUNGUAL ONYCHOMYCOSIS (PSO):

It is relatively uncommon type; organisms invade the nail via proximal nail fold through the cuticle area, penetrating newly formed area and migrate distally. Periungual inflammation is marked and painful, in some cases associated with purulent discharge. These patients are frequently misdiagnosed as bacterial having bacterial infections. It is increasingly reported in AIDS patients. Most common species involved is Candida.

3**. WHITE SUPERFICIAL ONYCHOMYCOSIS (WSO):**

This is surface infection of nail primarily when fungi invade superficial layers of nail plate directly and is caused by T. mentagrophuytes.

4. TOTAL DYSTROPHIC ONYCHOMYCOSIS (TDO):

There is total destruction of nail plate, which usually occurs as a result of infection by dermatophytes.

5. CANDIDAL ONYCHOMYCOSIS:

These are mostly caused by Candida albicans.

Figure 2: Distal and lateral subungual onchomycosis (DLSO)

Figure 3: Proximal subungual onychomycosis (PSO)

Figure: 4 Superficial White Onychomycosis (SWO)

Figure: 5 Total Dystrophic Onychomycosis (TDO)

Figure: 6 Candidal Onychomycosis with pseudoclubbing

Figure: 7 Onycholysis

Figure: 8 Sub ungual hyperkeratosis

LABORATORY DIAGNOSIS:

Detailed clinical examination of lesions can be done in bright light. Clinical lesions produced by these fungi are highly variable and closely resembles other non infectious nail disorders, making laboratory diagnosis and confirmation necessary.

Differential diagnosis for onychomycosis are psoriasis, lichen planus, contact dermatitis, traumatic onychodystrophies, paronychia congenita, nail bed tumour, yellow nail syndrome, idiopathic onycholysis, bacterial infections etc.[7]

Samples are divided into two portions. One part is used for culture and other portion is used for microscopy.

Microscopy:

40% KOH is used.

Modification of KOH for rapid visualisation of fungus:

(i) Addition of superchrome parker blue-black ink or some comparable substance to KOH solution. The ink selectively colours the hyphae, making them more pronounced. But it may take longer time to stain.

(ii) Addition of 36% dimethylsulphoxide (DMSO) to 20% or 40% KOH aids in penetration of specimen but over-digestion is a problem and the specimen has to be examined within 1-2 hour. Dimethylacetamide or dimethylformamide are used as alternatives.

(iii) Addition of 10-25% glycerine to KOH preparation to delay crystallization of KOH, degradation of fungus and rapid dehydration.

An alternative clearing agent, which does not require warming of specimen is 20% sodium sulphide solution.

Cultural isolation:

Samples are inoculated into two sets of culture media – Sabouraud's dextrose agar with chloramphenicol and Sabouraud's dextrose agar with chloramphenicol and cycloheximide. Tubes are incubated at 25^0 - 30^0C and at 37^0C. Most of the dermatophytes and non dermatophytic moulds will grow at the optimum temperature of $< 30^0$ C. Candida spp will grow at 37^0C. Cycloheximide inhibits the growth of most of the saprophytic fungi without inhibiting the growth of dermatophytes. Therefore Sabouraud's dextrose agar with chloramphenicol and cycloheximide medium is used as primary media for the isolation of dematophytes.

Routine incubation has traditionally been done for four weeks. Cultures should be examined at least every 2 – 3 days for the first two weeks and weekly thereafter.

1. Sabouraud's dextrose agar with chloramphenicol:

Sabouraud's dextrose agar with chloramphenicol (0.05%) inhibits the growth of bacteria without deterring the growth of filamentous fungi and yeast

2. Sabouraud's dextrose agar with chloramphenicol and cycloheximide:

The standard medium for growing dermatophyte is Sabouraud's dextrose agar containing chloramphenicol and cycloheximide, which inhibit bacteria and saprophytic fungi respectively. The cycloheximide (Actidione) in a concentration of 0.5 mg per ml suppresses the growth of most saprophytic fungi such as Scytalidium, Hendersonula, Aspergillus, Candida species without deterring the growth of dermatophytes. The various antibacterial antibiotics used include chloramphenicol (0.05 mg/ml) or gentamicin (0.1 mg/ml).

3. Potato dextrose agar:

It induces sporulation in filamentous fungi and can be used in slide cultures and antifungal susceptibility testing.

Identification by genus and species level:

Isolated fungi are identified the following methods

1. Colony morphology (obverse and reverse sides):

(i) Colony obverse:

The colour (white, pearl, ivory etc), texture of the surface (glabrous or waxy, powdery, granular, suede-like velvety, downy or fluffy).

(ii) Colony reverse:

Presence or absence of pigment, whether diffusing into the medium or not and topography (flat, raised, heaped) and rate of growth.

2. Lactophenol cotton blue mount (tease mount)

3. Slide culture technique

4. Cellophane or adhesive tape method

5. Gram staining from culture:

Candida spp seen as Gram positive budding yeast cells

6. Germ tube test:

This test is done to identify the Candida albicans species. Colonies of candida species are mixed with 0.5 ml of human serum or other suitable colloid in a sterile test tube. The tube is incubated at 37^0C for 2-3 hours. A drop of this mixture is kept over a microscope slide. It's covered with coverslip and examined for the production of germ tube – tube like projections from the yeast cells with out any constriction at the base. (Constriction at the base is seen in pseudo hyphae production). Germ tubes are true hyphal extensions. However 5% of Candida albicans will not produce the germ tube formation.

7. Physiological parameters:

Because of inconsistency in the development of several of the characteristic morphological features by these fungi and also due to infrequent

production of reproductive forms, some physiological criteria are often relied upon for a definite identification. These include

i) **Urease Test:**

A distinction between T. rubrum and T. mentagrophytes (particularly slow pigment producing varieties) is achieved by testing the strain for urease production. Both Christensen's urea agar and broth may be used. The test is positive in T. mentagrophytes and negative in T. rubrum.

ii) Pigment Production:

Generally T. rubrum isolates produce a dark cherry red colour under the colony. Confusion exists in differentiation of many slow pigment producing varieties of this species and similar isolates of T. mentagrophytes. For this purpose, stimulation of pigment production has been achieved by growing them on potato-dextrose agar and cornmeal dextrose agar media.

iii) Hair perforation Test:

The test was devised to distinguish between atypical isolates of T. mentagrophytes and T. rubrum. T. mentagrophytes produce hair perforating organs that penetrate hair radially and cause wedge shaped perforation where as T. rubrum do not. A sterile filter paper strip is placed into bottom of petridish with sterile distilled water. A small portion of sterilized prepubertal or infant hair is added into water. 5-6 drops of1% yeast extract is added to speed up reaction. Inoculate colony directly on hair and incubate at 25˚C for four weeks. Observe hair by making a wet smear for presence of conical perforations of hair shaft.[36]

TREATMENT OF ONYCHOMYCOSIS:

Antifungal agents:

Anti fungal antibiotics: Griseofulvin

It inhibits fungal mitosis by interference with polymerized microtubule and spindle formation in dividing cells. It is a fungi static drug.[31]

Allylamines:

These agents selectively inhibit the key enzyme squalene epoxidase which is required for fungal ergosterol biosynthesis and leads to accumulation of squalene which leads to weakening of cell membrane and cell death. Terbinafine is an allylamine, having high potency against nail infections shown by its very low MIC values. The fungicidal property, combined with its ability to penetrate rapidly into the nail plate, making it as a short course treatment drug which is feasible with a low potential for relapse.

Azoles:

The principal mode of action of the azole is the inhibition of cytochrome P-450 dependent C14 demethylation step. The important azoles used in the treatment of onychomycosis are ketoconazole and the newer triazoles fluconazole, itraconazole, voriconazole. Ketoconazole carries the risk of hepatotoxicity.

Topical antifungal agents:

They are used in fungal nail infections along with systemic agents. Ciclopiroxolamine 8% nail lacquer, amorolfine 5% nail lacquer and topical acidified nitrite.[37, 38]

Among oral antifungal agents, terbinafine is the drug of choice for tinea unguium. It is less effective against non dermatophytic infections, including Candida species and moulds. Fluconazole has a broad antifungal spectrum against dermatophytes, non dermatophytic moulds and Candida species particularly when used in pulse therapy. Itraconazole is the most

effective drug for the treatment of candidal onychomycosis. Onychomycosis in children is better treated by topical antifungal agents and are used as the first choice of treatment.[39]

ANTI FUNGAL SUSCEPTIBILITY TESTING:

The antifungal susceptibility is performed to provide the clinician to select an appropriate antifungal agent for treating a particular fungal infection. [31] However, these testing are not recommended as routine procedure.

The National Committee for Clinical Laboratory Standards (NCCLS) M38 - A and M27 – A which describes the standard parameters for testing MIC of established agents against filamentous fungi and yeasts respectively. M38 – A has latter been modified for testing dermatophytes M-38-P with several important factors, such as temperature (28 versus 35ºC) and time of incubation (4 to 10 days versus 21 to 72 hours). [40]

In vitro susceptibility testing of fungi, is influenced by a number of factors such as inoculum size, culture medium composition, pH, and duration and temperature of incubation and MIC end point determination. The trailing end point observed with the azoles is another major problem encountered in the susceptibility testing of the fungi. [31]

Antifungal susceptibility testing is receiving increased attention with the advent of newer anti fungal drugs. However susceptility testing of filamentous fungi is not as advanced as susceptibility testing of Yeasts. In vitro susceptibility tests should provide a reliable measure of relative activity of the anti fungal agent, correlate with in vivo activity and predict the likely outcome of the therapy. [41] Several studies have attempted to correlate the MIC results with outcome.

The retrospective nature of the studies, the documented variability of the nonstandardized in vitro methods and the difficulty in defining mycoses and their responses to therapy are responsible for this status. [42]

Other methods that have been used for performing anti fungal susceptibility testing are disk diffusion method, Etest, Fungi test, spectrophotometric methods and flowcytometry. [31]

MATERIALS AND METHODS

STUDY DESIGN: Prospective cohort study

The present study was conducted at Department of Microbiology at Coimbatore Medical College and Hospital, Coimbatore.

STUDY PERIOD:

Over a period of 15 months from April 2010 to June 2011.

SAMPLE SPECIFICATION:

Nail clippings, nail scrapings and sub ungual scrapings were collected from eighty five patients who attended the Dermatology outpatient Department at Coimbatore Medical College Hospital, Coimbatore.

INCLUSION CRITERIA:

All patients with clinically diagnosed onychomycosis, irrespective of age and sex were included in this study.

EXCLUSION CRITERIA:

All patients who are under treatment with antifungal drugs in the previous one month period were excluded from the study.

STUDY OF SELECTED CASES:

The selected cases were studied as per the proforma enclosed. A detailed history of selected cases was taken in relation to name, age, sex, address, occupation, duration of illness, associated skin and systemic infections.

After the detailed history, clinical examination of patient was made in good light which included site of nail involvement, number of nail units involved and the clinical type.

METHODOLOGY:

SPECIMEN COLLECTION:

The affected area of the nail was thoroughly cleaned with 70% alcohol to remove surface contaminants .The alcohol was allowed to dry by evaporation. The nail clippings and scrapings were taken by using sterile 15 No scalpel blade, from the advancing edges of diseased parts of the nails depending on the clinical type.[34]The scalpel blades were sterilised in autoclave at 121^{o}C in 15 lbs for 15 minutes.

TRANSPORT OF SAMPLES:

The samples were collected and transported in a sterile dry container to the laboratory.

PROCESSING OF SPECIMENS:

Direct microscopic examination:

Part of nail samples were immersed in 40% KOH and examined under low and high power of microscope after 24 – 48hrs for the presence of septate branched fungal hyphae / yeast cells

Modification of 40% KOH with DMSO:

Part of nail samples were immersed in solution of 40% KOH with DMSO and examined after one hour. The composition and preparation of the solution is attached in appendices. This method eliminated the need for waiting microscopic results for 24 – 48 hrs.

Culture:

After direct microscopic examination, irrespective of demonstration of fungal elements, the nail fragments were inoculated onto two sets of culture media, one containing Sabouraud's dextrose agar with 0.05% chloramphenicol, other containing Sabouraud's dextrose agar with 0.05% chloramphenicol and 0.5% cycloheximide.

One set of culture media was incubated at 37^{o}C and another set at 25 -30^{o}C for a period of four weeks. Culture slopes were examined for fungal growth daily in the first week and twice weekly from 2nd week onwards

Definitive diagnosis of non dermatophytic invasion of nails was done by

"WALSE AND ENGLISH" criteria (1966) [2, 50], i.e.

1. Microscopy positive for fungal elements

And

Culture also positive for NDM with the absence of concomitant dermatophytic growth with at least four to five inocula give rise to same growth from same specimen inoculated at different places on culture media.

Fungal isolates were identified based on colony morphology, pigmentation, microscopy (LPCB), slide culture, urease test (to differentiate T.mentagrophyte (positive) and T.rubrum (negative))

Macroscopic examination of culture:

The growth on Sabouraud's dextrose agar was examined to study the colony morphology based on following characteristics.

1. **Colony characters on obverse**:

The colour and consistency of colonies were studied. Regarding dermatophytes and yeasts growth were present on both SDA ie (chloramphenicol with/without cycloheximide) slopes. Where as NDM were grown on SDA slope without cycloheximide with the exception of penicillium spp which was cycloheximide tolerant.

Observation:

- Dermatophytes produced cottony white (T.mentagrophyte) and granular tan coloured (T.rubrum) colonies.

- Penicillium spp gave rise to green – grey suede like colonies.
- Aspergillus niger produced jet black colonies with pepper powdery growth.
- Aspergillus flavus gave rise to greenish velvety colonies.
- Candida spp produced creamy white smooth colonies.

2. **Colony characters on the reverse**:

Observed for the presence or absence of pigment, whether diffusing or not.

Observation:

- Few isolates of T.rubrum produced characteristic reddish brown pigment on reverse which diffused in to the medium.

Microscopic examination of culture:

a. LPCB mount:

A portion of aerial mycelium was removed and placed in a drop of lactophenol cotton blue over a glass slide. The matted mycelial mass was gently teased with a pair of teasing needles and the cover-slip was placed on it. The tease mount was observed under low and high power objective of microscope, for the presence of hyphae, macroconidia, microconidia and other accessory structures of vegetative hyphae and the characters of each was noted.

b. Slide culture:

Preparation of the petridish for slide culture:

Sterile petridish was taken and a piece of filter paper was cut according to the shape of petridish and placed inside the petridish. A 'V' tube was placed on the filter paper and a clean glass slide was placed over the 'V' tube and 1-2 cover-slips were placed inside. Petridish was closed with the lid, wrapped with paper and sterilized using hot air oven.

Procedure:

Blocks of (1 x 2 cms) Sabouraud's dextrose agar and corn meal agar were cut using sterile scalpel blade. One such block from both was transferred on to glass slide supported on 'V' tube in a petridish. Corners of the agar block were inoculated with the fungal colony using spud. A cover-slip was placed on surface of agar block using sterile forceps. Little amount of sterile distilled water was added to the petridish and the lid closed and incubated at room temperature. After each day the slide was taken out of petridish and examined under microscope without disturbing the cover-slip.

When the sporulation was well evident, cover-slip was carefully removed from the agar block and placed on a drop of lactophenol cotton blue stain taken on a separate glass slide.

One or two drops of lactophenol cotton blue were placed on the slide with fungal growth and cover-slip placed on it. Both the slides were examined under microscope. Character of hyphae, size, shape and arrangement of macroconidia, microconidia and other accessory vegetative structures were studied.

Advantage of slide culture over tease mount technique is that it allows the study of microscopic features of fungus preserving the continuity between the hyphae, microconidia and macroconidia.

c. Urease test:

This test was done on Christensens'urea medium. This was done to diffentiate T.mentagrophytes from T.rubrum. T,mentagrophytes strains, hydrolyse urea and the medium becomes deep red while T.rubrum does not hydrolyse urea.

CANDIDA SPECIES IDENTIFICATION:

a. Colony appearance:

Creamy white, smooth moist colonies were observed on SDA with chloramphenicol slope.

b. Microscopy – Gram stain:

Thin smear was made from the culture growth on a clean, grease free glass slide, stained with Grams stain, air dried and examined under microscope for the presence of Gram positive budding yeast cells.

c. Germ tube test:

Germ tube test was done for the presumptive identification of Candida albicans species. 0.5ml of human serum was taken in a sterile test tube. To that added small portion of Candida culture and incubated at $37^{o}C$ for 2- 4 hrs. A drop of this suspension was placed on a slide. A coverslip was put and examined under low power, high power of microscope for the presence of germ tubes. These are tube like extensions from the yeast cells, without any constrictions at their point of origin (base).

ANTI FUNGAL SUSCEPTIBILITY TESTING OF FILAMENTOUS FUNGI AND YEAST BY MODIFIED CLSI MICRO BROTH DILUTION METHOD (M38 – A & M27 – A)

I. Test medium

RPMI-1640 (with glutamine, without bicarbonate, and with a pH indicator) is the medium recommended for the susceptibility testing of filamentous fungi. The medium should be buffered with MOPS (0.164 mol/L).

Procedure for making RPMI-1640 medium:

• 10.4 g powdered RPMI-1640 medium (HIMEDIA).

• 34.53 g MOPS buffer (HIMEDIA).

1) Dissolved powdered medium in 900 mL of distilled water.

2) Added MOPS, and stirred until dissolved.

3) Adjusted the pH to 7.0 at 25 using 10 or 1 mol/L sodium hydroxide while stirring.

4) Added additional water to bring the medium to a final volume of 1 L.

II. Anti fungal stock solution and Drug dilution preparation:

Anti fungal agents used: (SIGMA ALDRICH)

Powder forms of,

Griseofulvin (solvent: DMSO)

Ketoconazole (solvent: DMSO)

Fluconazole (solvent: Distilled water)

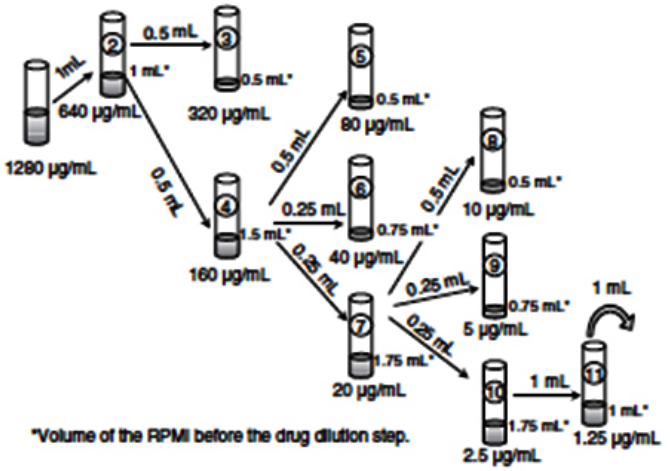
Terbinafine (solvent: DMSO)

Stock solution preparation:

a) For water **soluble** drugs: FLUCONAZOLE
• 1280µg/ml

b) For water **insoluble** drugs:GRISEOFULVIN, KETOCONAZOLE and TERBINAFINE
• 1600µg/ml

Drug dilution preparation: For water soluble drugs: FLUCONAZOLE



i) Labelled 10 test tubes 2–11.

ii) Added the appropriate amounts of sterile RPMI to each tube as follows:

• Added 1 mL of RPMI to tubes 2 and 11.

• Added 0.5 mL of RPMI to tubes 3, 5, and 8.

• Added 0.75 mL of RPMI to tubes 6 and 9.

• Added 1.5 mL of RPMI to tube 4.

• Added 1.75 mL of RPMI to tubes 7 and 10.

• Finally discarded 1mL from tube 11.

• When the dilutions have been prepared, each tube contains 1 mL.

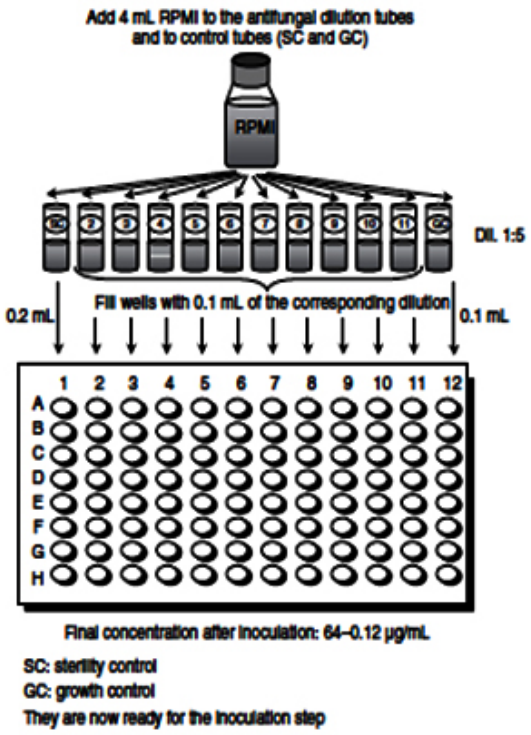
• Mixed well with a vortex mixer before each dilution step.

Preparation of micro dilution trays for water-soluble antifungal agents:

i) Placed the tubes containing the drug dilutions (1 mL each) in an organized fashion, going from the highest to the lowest drug concentration.

ii) Prepared a 1:5 dilution by adding 4 mL of RPMI to each drug dilution tube and mix well with a vortex mixer

iii) Using a dispensing device, transferred 0.1 mL from each of the drug dilution tubes to the appropriate wells of each microtiter tray.



• **The concentration of the antifungal agent in each well is now 2 times the concentration needed after this step.**

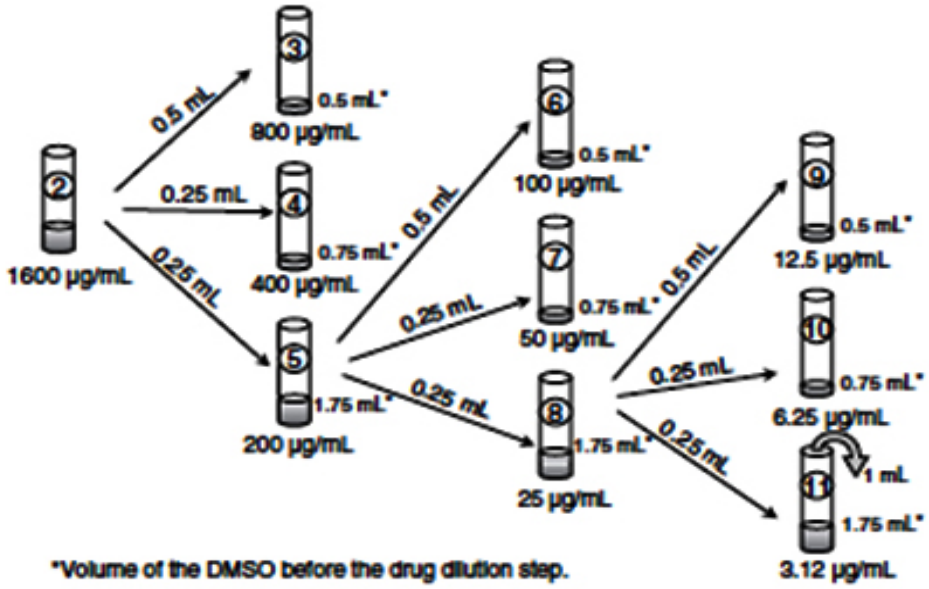
iv) Added 0.2 mL of RPMI to the wells in column 1 and 0.1 mL to wells in column 12. These wells will serve as the negative (sterility) and positive (growth) control wells, respectively.

Drug dilution preparation: for water insoluble drugs: GRISEOFULVIN, KETOCONAZOLE and TERBINAFINE

• The dilutions of water-insoluble antifungal agents must be prepared by using dimethyl sulfoxide (DMSO)

• It is important to prepare the stock solution and drug dilutions in the solvent to avoid dilution artefacts

a)



Labelled 9 (12 × 75 mm) tubes 3–11.

b) Added appropriate amounts of DMSO to each tube as follows:

• Added 0.5 mL of DMSO to tubes 3, 6, and 9

• Added 0.75 mL of DMSO to tubes 4, 7, and 10

• Added 1.75 mL of DMSO to tubes 5, 8, and 11

c) Labelled the stock solution tube (1,600 µg/mL) as tube 2

d) Transferred from tube 2, 0.5 mL to tube 3 and 0.25 mL to tubes 4 and 5

e) Transferred from tube 5, 0.5 mL to tube 6 and 0.25 mL to tubes 7 and 8

f) Transferred from tube 8, 0.5 mL to tube 9 and 0.25 mL to tubes 10 and 11

g) Finally, discarded 1 mL from tube 11

h) When dilutions have been prepared, all the tubes contain 1 mL.

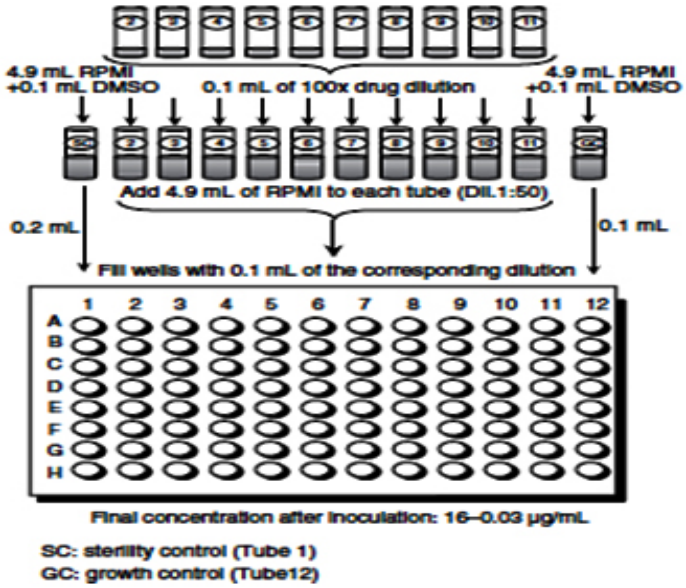
Preparation of microdilution trays for water-insoluble antifungal agents

a) Placed the tubes containing the drug dilutions (1 mL) in an organized fashion, from the highest to the lowest drug concentration and labelled a row of 10 tubes with the appropriate drug concentration.

b) Prepared a 1:50 dilution by mixing 4.9 mL of RPMI with 0.1 mL from each drug dilution tube; mixed well with a vortex mixer.

c) Using a dispensing device, transferred 0.1 mL from each of the final drug dilution tubes to the appropriate well in each microtiter tray.

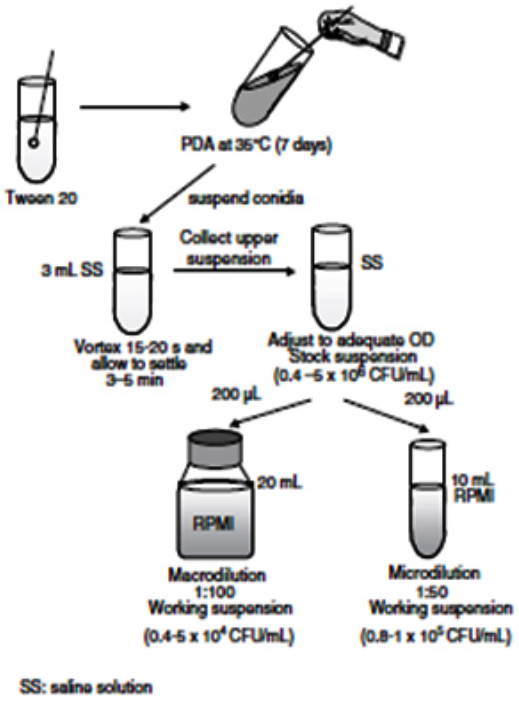
d) Added 0.2 mL of RPMI plus 2% DMSO to column 1 of the microtiter tray and 0.1 mL of RPMI plus 2% DMSO to column 12. These two wells will serve as the negative (sterility) and positive (growth control) control wells, respectively, for the microtiter tray.



• **The concentration of the antifungal agents in the wells is now 2 times the concentration needed.**

• **The concentration of DMSO in the wells is now 2%.**

iii) Inoculum preparation – For filamentous fungi:



a) Inoculum must be prepared from a conidium or sporangiospore suspension obtained from 7-day cultures grown on potato dextrose agar at 25°C–28°C. Some isolates need longer incubation (more than 7 days) to produce conidia.

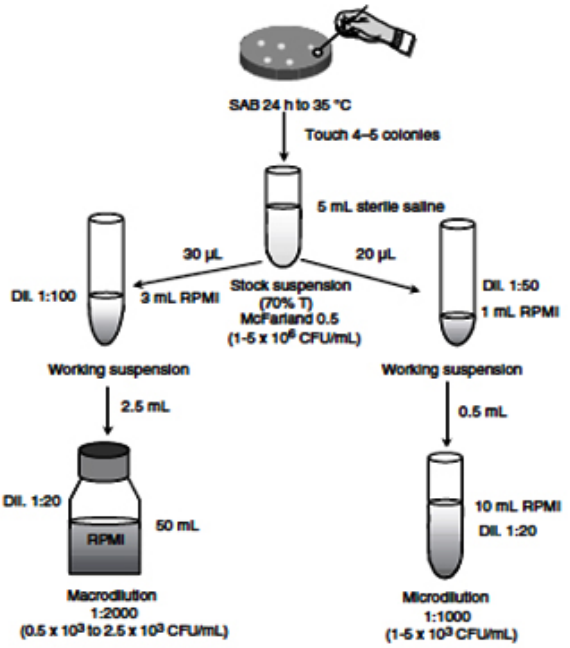
b) Recovered conidia by wetting surface of the colony with sterile normal saline and transferred the loopful of conidia into 3 mL of sterile saline.

c) Vortex the conidia suspension vigorously for 15–20 s to prevent clumping of the spores.

d) Allowed the heavy particles to settle for 3–5 min, and then transferred the upper suspension to a sterile tube and adjusted the turbidity using 0.5McFarland standard.

e) Prepared a working suspension by diluting 1:50 of the conidia stock suspension in the standard medium (200µL of stock suspension into 10mL of RPMI), mixed well with a vortex mixer.

B) For Yeast:



iv) Inoculation of microtiter trays:

Fill each row of the microtiter tray from wells 2–12 with 0.1 mL of the working inoculum suspension using a single or a multichannel pipette

Incubation

MIC trays were incubated in incubator at 35°C for 46–50 h for most of the opportunistic moulds and yeast

v) Reading & Interpretation:

Visual reading:

1) Removed the trays from the incubator.

2) Placed the tray on a reading device (mirror reader)

3) The growth control well must have sufficient growth.

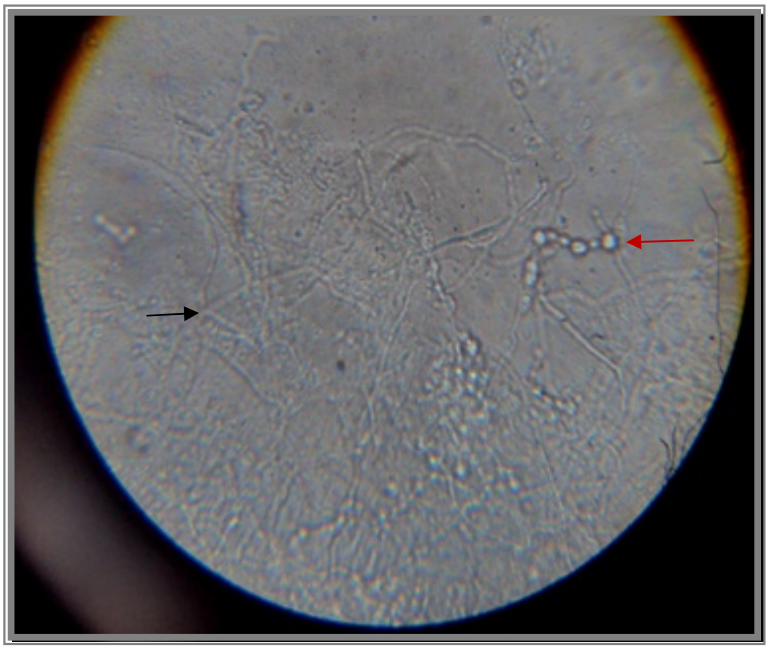
4) If adequate growth is not present, the trays were reincubated. Adequate growth will depend on the species being tested.

5) Determine the MIC endpoints – MIC50 & MIC90.

MIC50 - the lowest drug concentration that prevents any discernible growth (visually clear) in 50% of the strains when compared to the GC strain.

MIC90 - the lowest drug concentration that prevents any discernible growth (visually clear) in 90% of the strains when compared to the GC strain.

Figure9: MICROSCOPY (40% KOH)



→ Shows Septate hyphae

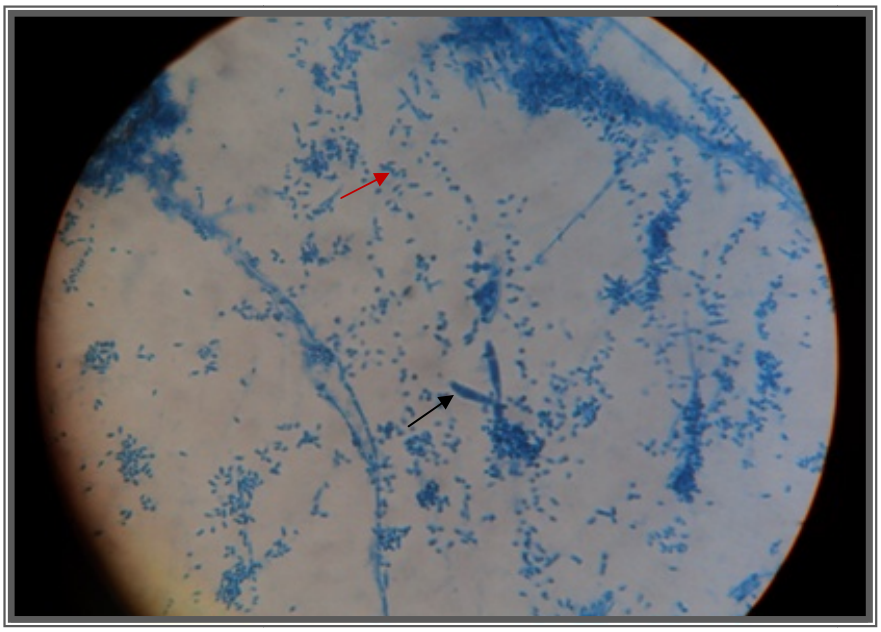
→ Shows Chlamydospore

TRICHOPHYTON RUBRUM

Figure10: Culture (Obverse & Reverse)

Shows Redpigment on Reverse

Figure11: LPCB mount



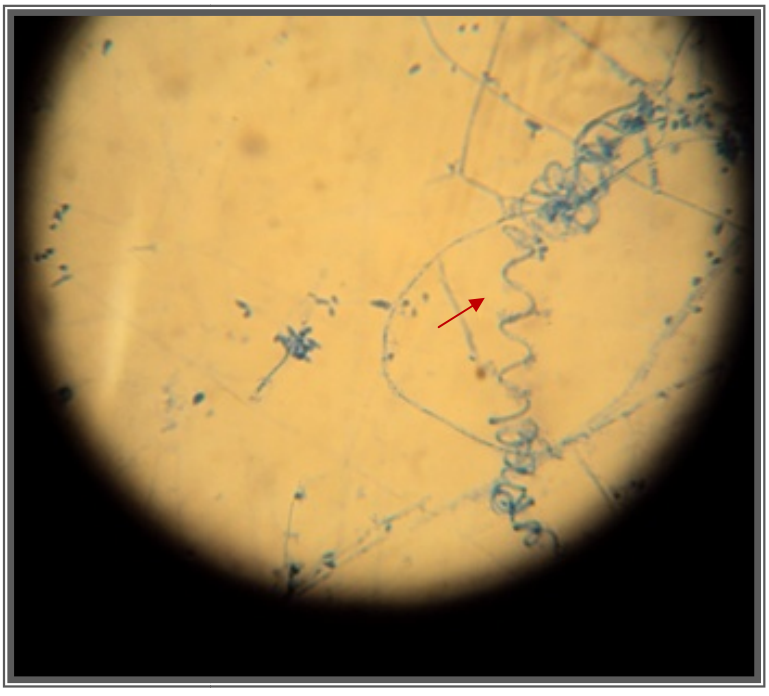
Shows Microconidia

Shows Macroconidia

TRICHOPHYTON MENTAGROPHYTE

Figure12: Culture (Colony Obverse & Reverse)

Figure13: LPCB mount

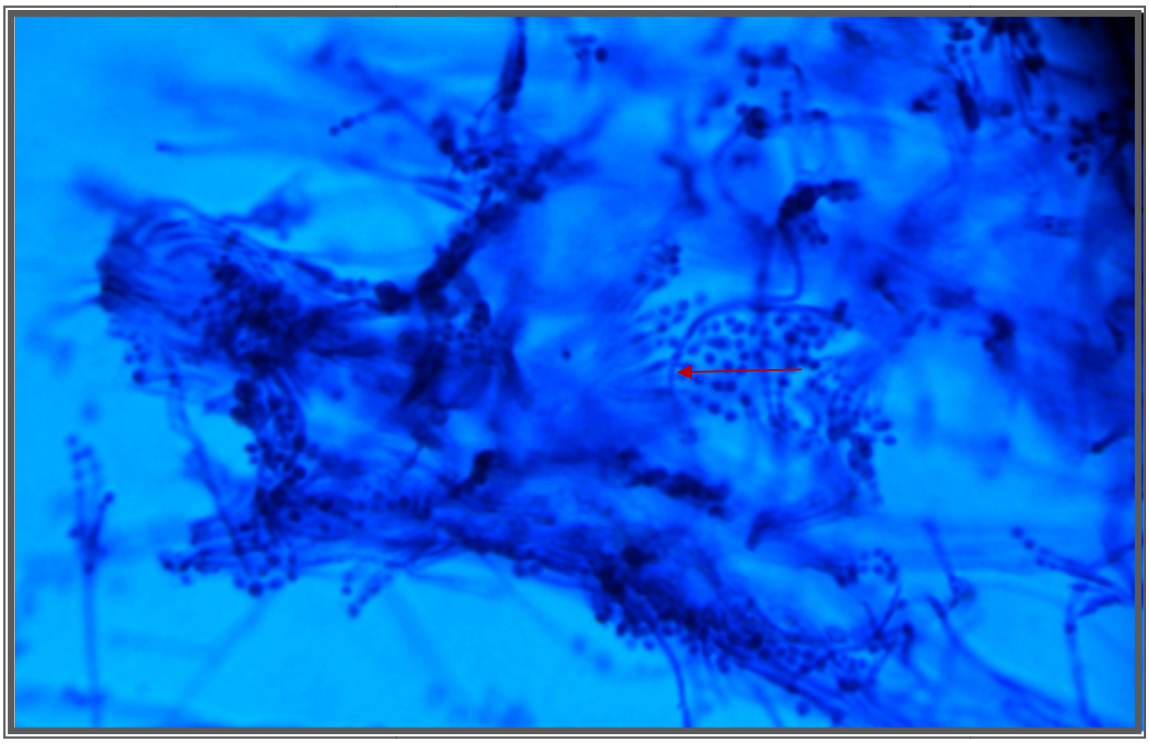


Shows Spiral hyphae

PENICILLIUM SPECIES

Figure14: Culture (Colony Obverse & Reverse)

Figure15: LPCB mount

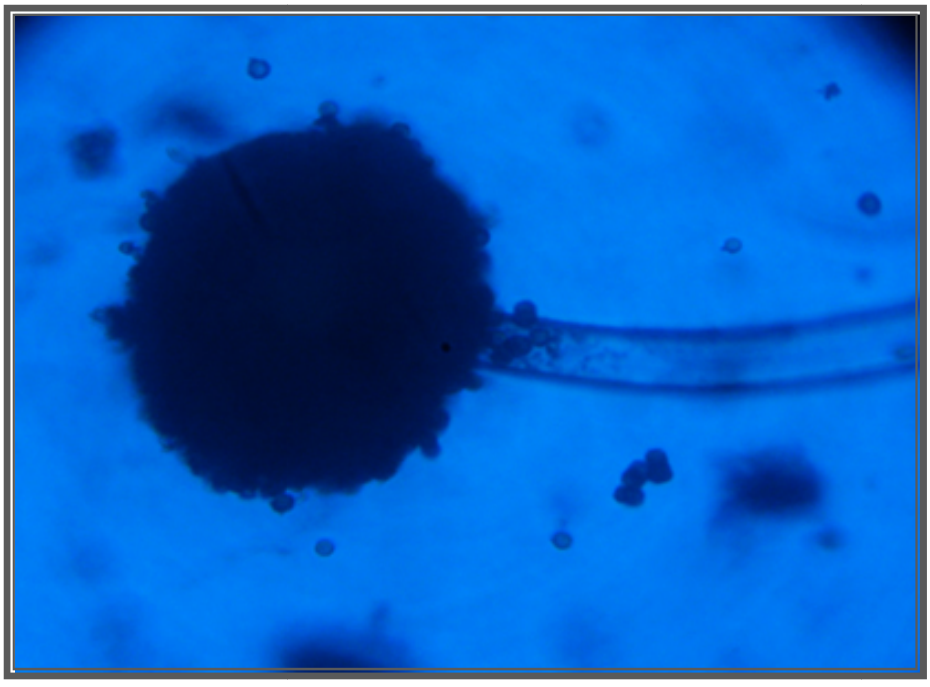


Shows brush shaped conidia

ASPERGILLUS NIGER

Figure16: Culture (Colony Obverse & Reverse)

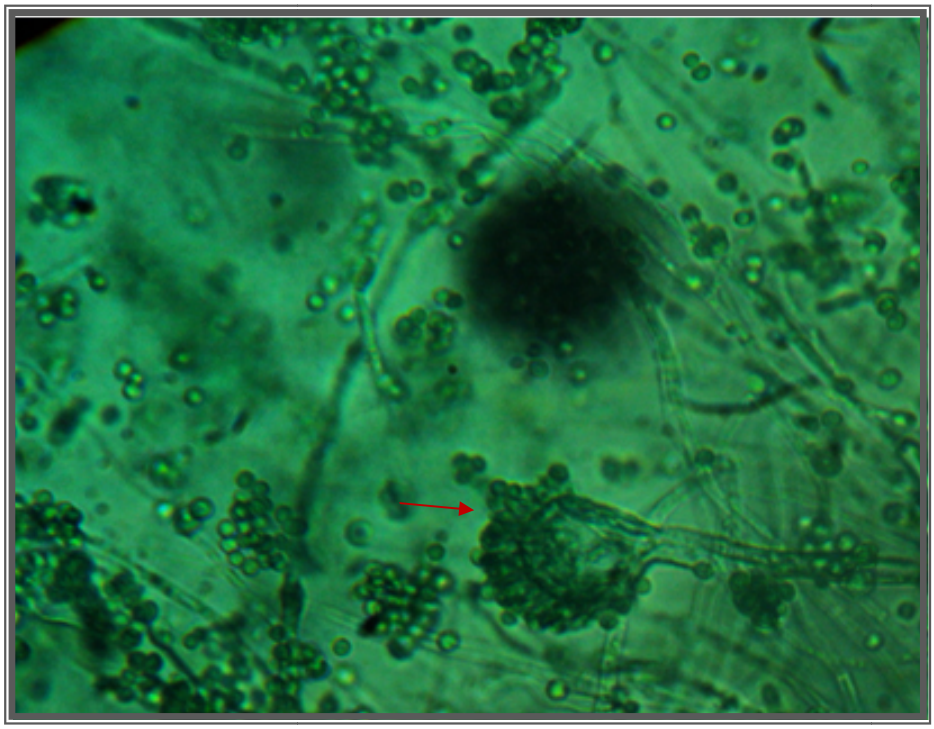
Figure17: LPCB mount



ASPERGILLUS FLAVUS

Figure18: Culture (Colony Obverse & Reverse)

Figure19: LPCB mount

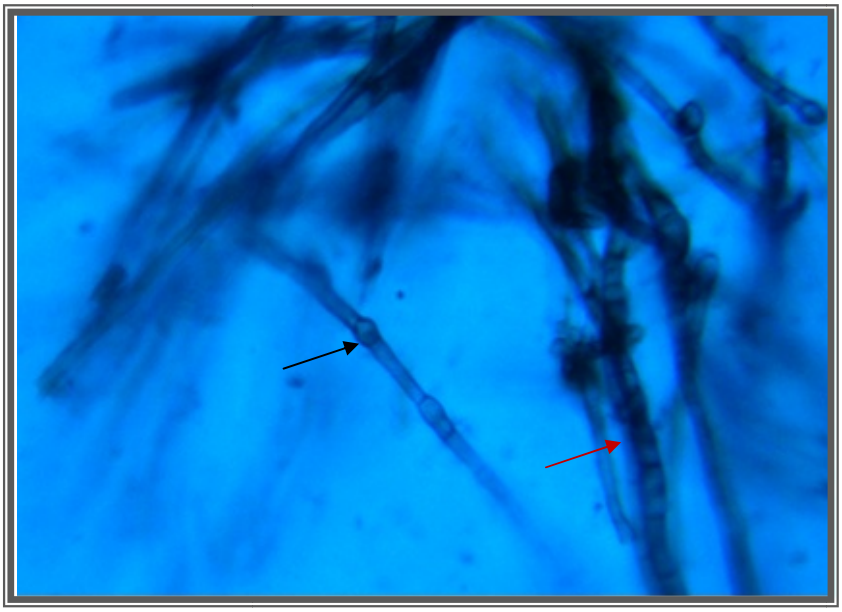


Shows Single row of phialides with conidia covering 2/3 of vesicle

CLADOSPORIUM SPECIES

Figure20: Culture

Figure21: LPCB mount



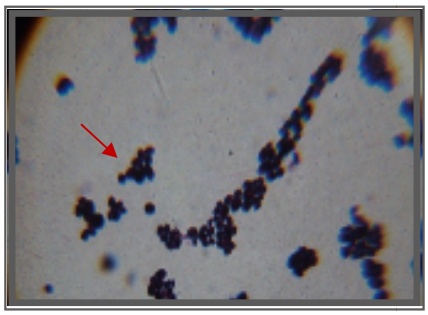
Shows Pigmented hyphae

Shows Intercalary swelling

CANDIDA ALBICANS

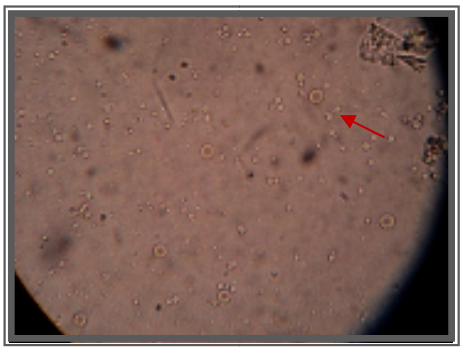
Figure22: Culture

Figure23: Gram stain



Shows Gram+ve yeast cells

Figure24: Germ tube test



Shows Germ tube

Figure25: Slide culture

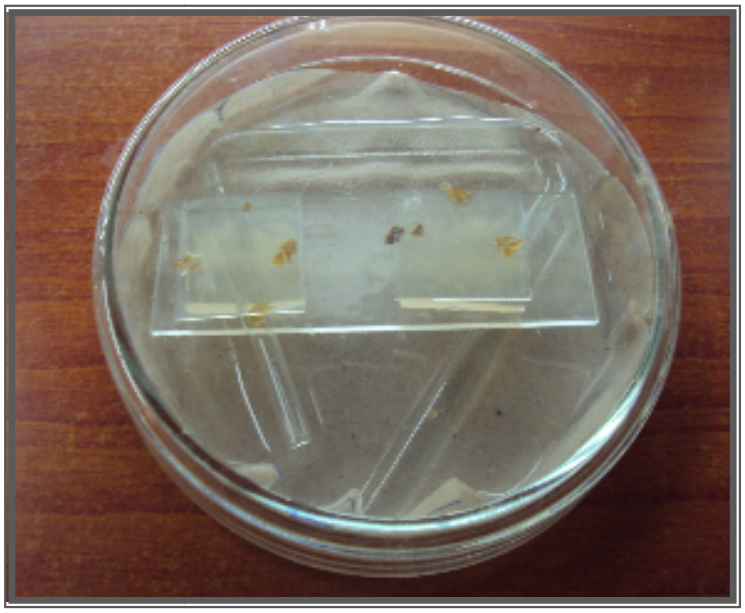


Figure26: Urease test – T.mentagrophyte (+ve) & T.rubrum (-ve)

Figure28: Antifungal susceptibility testing- MIC90 of Griseofulvin

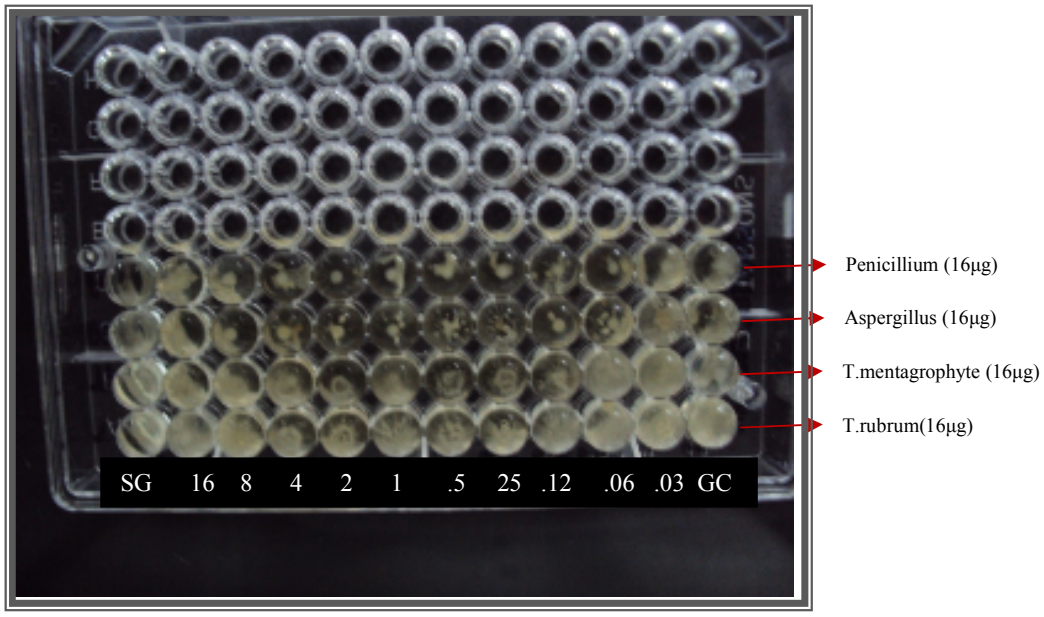


Figure29: Antifungal susceptibility testing – MIC90 of Ketoconazole

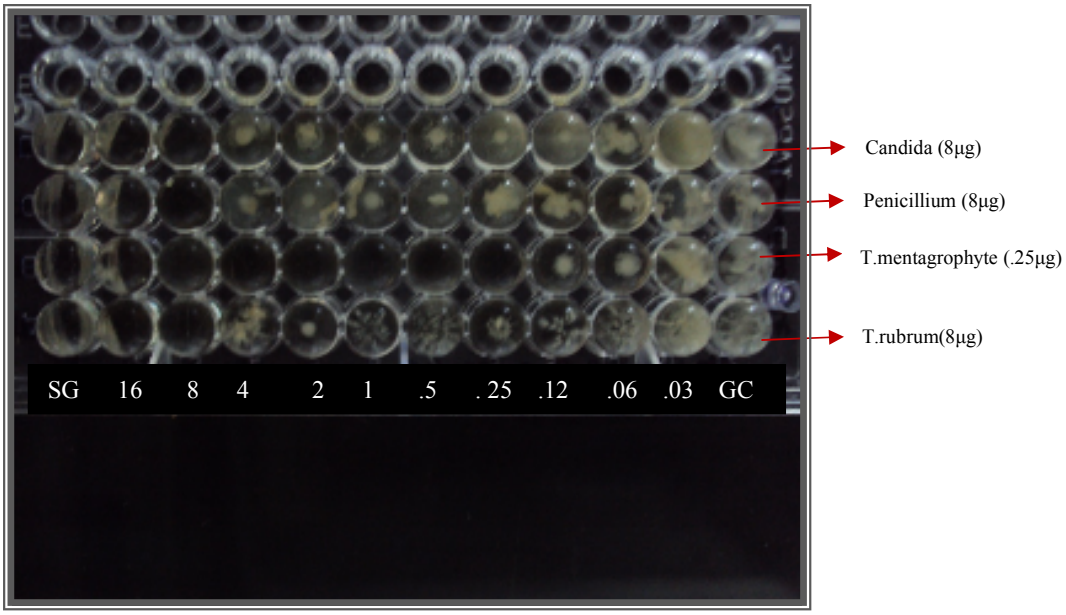


Figure30: Antifungal susceptibility testing – MIC90 of Fluconazole

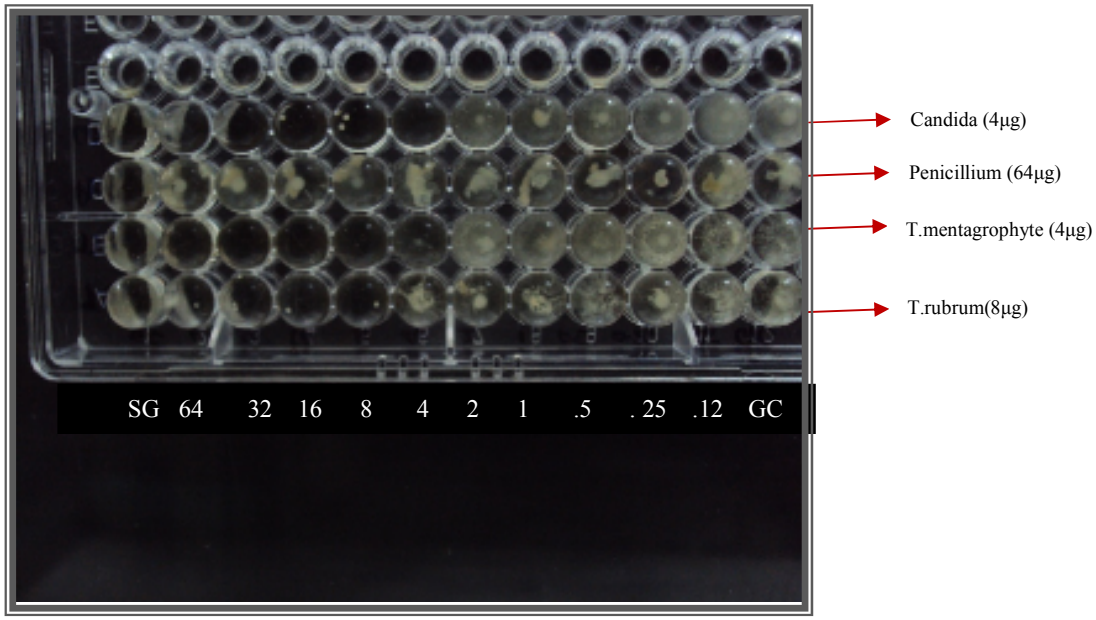
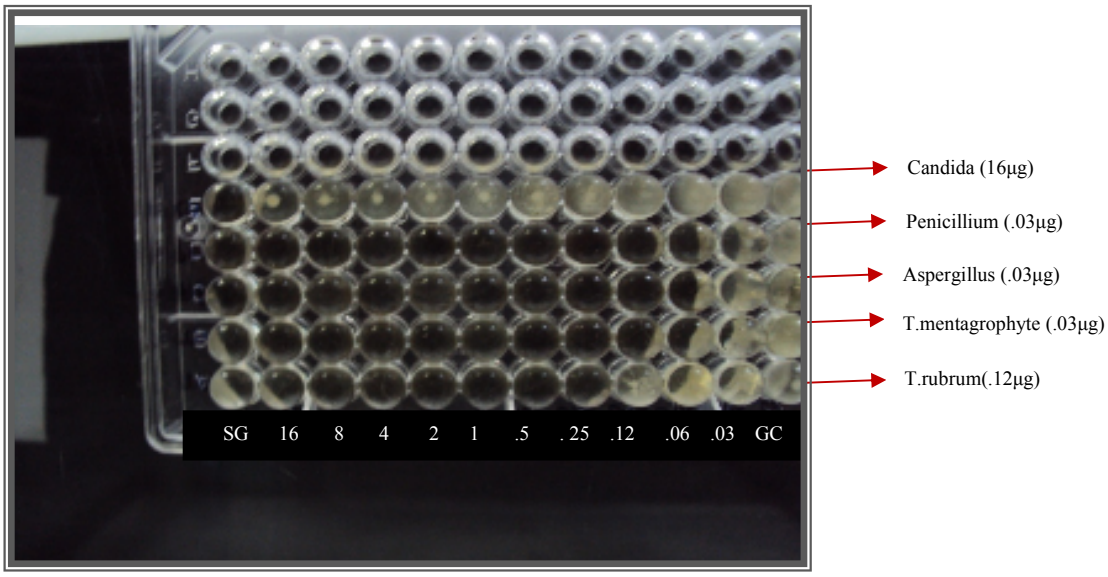


Figure31: Antifungal susceptibility testing – MIC90 of Terbinafine



RESULTS

During the study period, a total of 85 cases with the clinical diagnosis of onychomycosis were studied by direct microscopy and fungal culture techniques.

The statistical analysis of the variables was done by using SPSS software. P values were obtained from Pearson Chi Square test.

The age of the patients under study ranged from 6 – 65years. The commonest age group affected ranged from 41-50 years (28.30%) followed by 31-40years (18.18%) and 51-60years (18.18%). Onychomycosis was less common in the age group below 20years (4.7%). **(Ref: Table 1)**.

The male: female ratio was 2.7:1 with 62 (72.95%) cases of males and 23 (27.05%) cases of females. **(Ref: Table 2)**. The highest incidence among males was observed in the age group 41 – 50years (20 cases), while among females it was 51 – 60years (8 cases). **(Ref: Table 3)**.

Among all the age group, distal and lateral subungual onychomycosis (DLSO) was the commonest clinical pattern observed in 48cases (56.50%) followed by superficial white onychomycosis (SWO)12cases (14.1%), paronychia 10cases (11.8%) and total dystrophic onychomycosis (TDO) 9cases (10.6%). The less common clinical type was proximal subungual onychomycosis (PSO) 6cases (7%). **(Ref: Table 4 & 5)**.

Regarding the site of involvement the fingernails were commonly involved 60cases (70.59%) than toe nails 17cases (20%). Finger nails and toe nails both were involved in 8cases (9.41%). P value obtained for correlation of fingernail involvement and Paronychia type was 0.007. **(Ref: Table 5)**. The nail changes observed in these cases were discolouration (100%), subungual hyperkeratosis (45%), nail thickening (22%) and onycholysis (18%). **(Ref: Table 7)**

The majority of patients were farmers (34.11%) followed by office workers (25.88%) and house wives (24.71%). The P value derived was 0.01**(Ref: Table 6)**.

Small number of patients had other associated skin and systemic infections, (18.9%). Skin infections were tenia corporis 7cases (8.7%), Psoriasis 2cases (2.35%), chronic eczema 1case (1.2%) and Hansen's disease 1case (1.2%). Systemic diseases were diabetes mellitus (1.2%), Systemic hypertension (2.35%), chronic kidney disease with chronic renal failure (1.2%) and AIDS on ART (1.5%). The P value derived for T.corporis correlation was 0.05**(Ref: Tab 8)**.

Out of the 85 clinically diagnosed onychomycosis, 60 cases (70.58%) were positive by microscopy (i.e., 40% KOH) and/or culture. 25 cases (29.41%) were negative by both the techniques. Total culture positive cases were 46 (54.12%). Total KOH positive cases were 43 (50.59%). **(Ref: Table 9)**.

The two microscopic techniques (KOH alone & KOH with DMSO) were compared. The sensitivity of both the techniques was 50.6% and 48.2% respectively while the P values calculated were 0.294 and 0.588. **(Ref: Table 10)**

The commonest fungi isolated in onychomycosis were dermatophytes 24 (n =85, 28.2%) of which T.mentagrophyte was the commonest species 14/24 (16.5%) followed by T. rubrum 10/24 (11.8%). The non dermatophytic moulds (NDM) were the second common isolates, 13 (15.3%). Among the NDM, Penicillium species represented as the common species 9/13 (10.6%). Other non dermatophytic moulds isolated were Aspergillus niger 2/13 (2.4%), Aspergillus flavus 1/13 (1.2%) and Cladosporium species 1/13 (1.2%). The third common group obtained were yeasts, the species being Candida albicans 9 (10.6%). No growth was observed in 39 cases (45.9%). **(Ref: Table 11)**

The isolation rate was higher in males 33 (n = 46, 72%) than in females 13 (28%) for all the fungi with the exception of Penicillium species (9% in males, 11% in females) and Candida albicans (9% in males, 11% in females). It was also observed that finger nails yielded more number of isolates 35 (76%) than toe nails 8 (18%). The P value for Candida albicans isolate and the female patients was 0.085.

(Ref: Tab 12 & 13).

The fungal isolates were tested for the susceptibility of four anti fungal drugs namely griseofulvin, ketoconazole, fluconazole and terbinafine by CLSI M38 A (filamentous fungi), M27 A2 (yeasts) broth micro dilution methods. All the isolates were resistant to griseofulvin with the MIC50 & MIC 90 as follows

T.mentagrophyte (n = 14): MIC50 – 8µg/ml and MIC90 – 16µg/ml

T.rubrum (n = 10): MIC50 - 4µg/ml and MIC90 – 16µg/ml

Aspergillus spp (n = 3): MIC50 – NIL and MIC90 - 16µg/ml

Penicillium spp (n = 9): MIC50 – NIL and MIC90 - 16µg/ml.

Candida albicans was not tested against griseofulvin because of its intrinsic resistance.

(Ref: Table 14).

The MIC50 & MIC 90 of ketoconazole as follows

T.mentagrophyte (n = 14): MIC50 – 0.12µg/ml and MIC90 – 0.25µg/ml

T.rubrum (n = 10): MIC50 - 4µg/ml and MIC90 – 8µg/ml

Penicillium spp (n = 9): MIC50 – 2µg/ml and MIC90 - 8µg/ml

Candida albicans (n = 9): MIC50 – 2µg/ml and MIC90 - 8µg/ml

Aspergillus species was not tested against ketoconazole because of its intrinsic resistance.

(Ref: Table 15).

The MIC50 & MIC 90 of fluconazole as follows

T.mentagrophyte (n = 14): MIC50 – 2µg/ml and MIC90 – 4µg/ml

T.rubrum (n = 10): MIC50 - 2µg/ml and MIC90 – 8µg/ml

Penicillium spp (n = 9): MIC50 – NIL and MIC90 - 64µg/ml (resistant)

Candida albicans (n = 9): MIC50 – 2µg/ml and MIC90 - 4µg/ml

Aspergillus species was not tested against fluconazole because of its insignificant in vivo activity. **(Ref: Table 16)**.

The MIC50 & MIC 90 of terbinafine as follows

T.mentagrophyte (n = 14): MIC50 – NIL and MIC90 – 0.03µg/ml

T.rubrum (n = 10): MIC50 – 0.06µg/ml and MIC90 – 0.12µg/ml

Aspergillus spp (n = 3): MIC50 – NIL and MIC90 – 0.03µg/ml

Penicillium spp (n = 9): MIC50 – NIL and MIC90 – 0.03µg/ml

Candida albicans (n = 9): MIC50 – 2µg/ml and MIC90 - 16µg/ml (most of the isolates were resistant). **(Ref: Table 17)**.

Table: 1 Age wise distribution of cases

Age	Frequency	Percentage
<10	1	01.20
11-20	3	03.50
21-30	14	16.50
31-40	16	18.80
41-50	24	28.30
51-60	16	18.80
>60	11	12.90
Total	85	100

Table 2: Sex wise distribution of cases

Sex	Frequency	Percentage	
Male	62	72.95	M/F Ratio
Female	23	27.05	2.7:1
Total	85	100	

Table 3: Age & Sex wise distribution of cases

Age	Sex		Total
	Male	Female	
<10	0	1	1
11-20	3	0	3
21-30	8	6	14
31-40	12	4	16
41-50	20	4	24
51-60	8	8	16
>60	9	2	11
Total	60	25	85

Table 4: Age & Sex wise distribution of clinical type

Clinical pattern	<10		11-20		21-30		31-40		41-50		51-60		>60	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
DLSO	-	-	1	-	6	1	7	3	12	2	4	5	7	-
PSO	-	-	-	-	1	1	2	-	-	-	-	2	-	-
SWO	-	1	1	-	1	-	-	1	2	-	2	1	1	2
TDO	-	-	-	-	-	1	1	-	5	-	1	-	1	-
Paronychia	-	-	1	-	-	3	2	-	1	2	1	-	-	-
Total	0	1	3	0	8	6	12	4	20	4	8	8	9	2

Table 5: Clinical types in relation to site of involvement

Clinical pattern	Finger nail	Toe nail	Both	Total	%	P value
DLSO	32	10	6	48	56.5%	-
PSO	4	2	-	6	7%	-
SWO	9	3	-	12	14.1%	-
TDO	6	1	2	9	10.6%	-
Paronychia	9	1	-	10	11.8%	0.007
Total (%)	60 (70.59)	17 (20)	8 (9.41)	85	-	-

Table 6: Clinical types in relation to occupation

Clinical type	Occupation					Total
	F	O	H	S	M	
DLSO	19	16	10	1	2	48
PSO	2	1	3	-	-	06
SWO	4	1	3	1	3	12
TDO	2	2	1	-	4	09
Paronychia	2	2	4	2	-	10
Total (%)	29 (34.11)	22 (25.88)	21 (24.71)	4 (4.71)	9 (10.59)	85
Chi Square value	31.729	-	-	-	-	-
P value	0.011	-	-	-	-	-

Table 7: Nail changes in Onychomychosis

Nail changes	N = 85 (%)
Discolouration	85 (100)
Subungual hyperkeratosis	38 (44.7)
Thickening	19 (22.35)
Onycholysis	15 (17.65)
Dystrophy	12 (14.12)
Paronychia	10 (11.76)

Table8: Associated skin and systemic diseases in onychomycosis

Disease	Number	%	Chi square value	P value
Tenia corporis	7	8.2%	13.588	0.059
Psoriasis	2	2.35%	-	-
Eczema	1	1.2%	-	-
Hansen's disease	1	1.2%	-	-
Diabetes mellitus	1	1.2%	-	-
SHT	2	2.35%	-	-
CKD with CRF	1	1.2%	-	-
HIV	1	1.2%	-	-
Total	16	18.9%	-	-

Table9: Microscopy versus culture in Onychomycosis

Microscopy (40% KOH)	Culture		Total (%) n = 85
	Positive (%)	Negative (%)	
Positive (%)	29 (34.12)	14 (16.47)	43 (50.59)
Negative (%)	17 (20)	25 (29.41)	42 (49.41)
Total (%)	46 (54.12)	39 (45.88)	85 (100)

Table 10: Microscopy – 40% KOH VS 40% KOH with DMSO

Technique	Positive (%)	Negative	Chi Square value	P value
40% KOH	43(50.6)	42 (49.4)	0.953	0.294
40% KOH & DMSO	41 (48.2)	44 (51.8)	0.329	0.588

Table 11: Fungal isolates obtained from nail samples

Group	Species	No	% (n= 85)	Total	Total %
Dermatophyte	T.mentagrophyte	14	16.5%	24	28.3%
	T.rubrum	10	11.8%		
NDM	Aspergillus niger	2	2.4%	13	15.3%
	Aspergillus flavus	1	1.2%		
	Cladosporium spp	1	1.2%		
	Penicillium spp	9	10.6%		
Yeast	Candida albicans	9	10.6%	9	10.6%
No growth	-	39	-	-	45.8%

Table 12: Culture results of onychomycosis according to gender

Group	Species	Male (%)	Female (%)	Total
Dermatophyte	T.mentagrophyte	12 (26%)	2 (4%)	14
	T.rubrum	9 (20%)	1 (2%)	10
NDM	Aspergillus niger	2 (4%)	-	2
	Aspergillus flavus	1 (2%)	-	1
	Cladosporium spp	1 (2%)	-	1
	Penicillium spp	4 (9%)	5 (10%)	9
Yeast	Candida albicans	4 (9%)	5 (10%)	9
Total (%) n = 46		33 (72%)	13 (28%)	46

Table 13: Culture results of onychomycosis according to site of involvement

Group	Species	Finger nail	Toe nail	Both	Total
Dermatophyte	T.mentagrophyte	8 (17.5%)	4 (9%)	2 (4%)	14
	T.rubrum	6 (13%)	3 (6.5%)	1 (2%)	10
NDM	Aspergillus niger	2 (4%)	-	-	2
	Aspergillus flavus	1 (2%)	-	-	1
	Cladosporium spp	1 (2%)	-	-	1
	Penicillium spp	8 (17.5%)	1 (2%)	-	9
Yeast	Candida albicans	9 (20%)	-	-	9
Total (%)n =46		35 (76%)	8 (17.5%)	3 (6.5%)	46

ANTIFUNGAL SUSCEPTIBILITY TESTING OF ISOLATES BY BROTH MICRODILUTION METHOD – MIC DETERMINATION

Table 14: MIC values of Griseofulvin for the isolates

Isolate	Concentration of drug (µg/ml)											
	MIC 50	MIC 90	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
T.mentagrophyte n = 14	8	16	-	-	-	-	-	-	-	1	2	11
T.rubrum n= 10	4	16	-	-	-	-	-	1	1	2	4	2
Aspergillus spp n = 3	-	16	-	-	-	-	-	-	-	-	-	3
Penicillium spp n = 9	-	16	-	-	-	-	-	-	-	-	-	9

Table 15: MIC values of Ketoconazole for the isolates

Isolate	Concentration of drug (µg/ml)											
	MIC 50	MIC 90	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
T.mentagrophyte n=14	0.12	0.25	-	3	4	4	1	2	-	-	-	-
T.rubrum n=10	4	8	-	-	-	-	-	-	1	5	4	-
Penicillium spp n = 9	2	8	-	-	-	-	-	1	4	1	2	1
Candida albicans n = 9	2	8	-	-	-	-	-	1	5	1	2	-

Table 16: MIC values of Fluconazole for the isolates

Isolate	Concentration of drug (µg/ml)											
	MIC 50	MIC 90	0.12	0.25	0.5	1	2	4	8	16	32	64
T.mentagrophyte n = 14	2	4	-	-	2	2	3	5	2	-	-	-
T.rubrum n= 10	2	8	-	-	-	-	5	1	3	1	-	-
Penicillium spp n = 9	-	64	-	-	-	-	-	-	-	-	1	8
Candida albicans n = 9	2	4	-	1	1	2	1	3	1	-	-	-

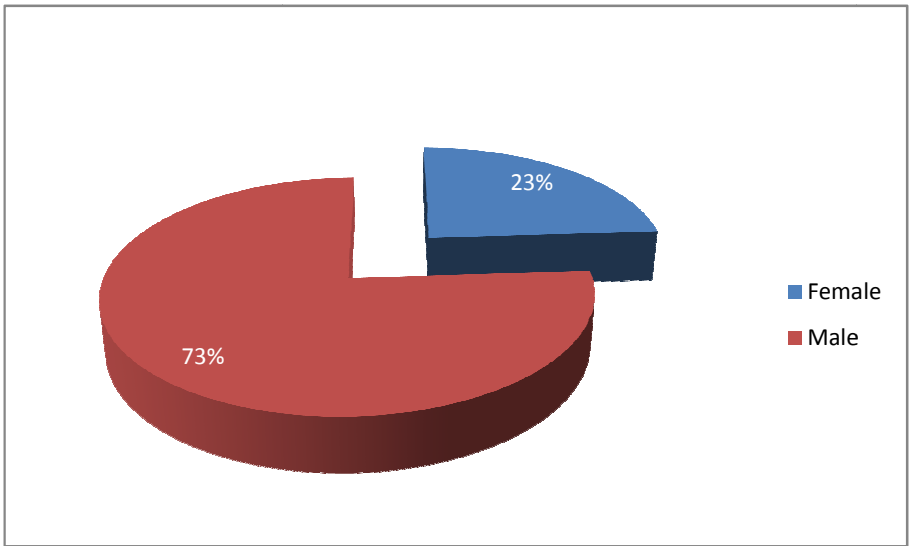
Table 17: MIC values of Terbinafine for the isolates

Isolate	Concentration of drug (µg/ml)											
	MIC 50	MIC 90	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
T.mentagrophyte n = 14	-	0.03	12	2	-	-	-	-	-	-	-	-
T.rubrum n= 10	0.06	0.12	1	4	4	1	-	-	-	-	-	-
Aspergillus spp n = 3	-	0.03	3	-	-	-	-	-	-	-	-	-
Penicillium spp n = 9	-	0.03	8	1	-	-	-	-	-	-	-	-
Candida albicans n=9	2	16	-	-	-	-	-	-	4	1	1	3

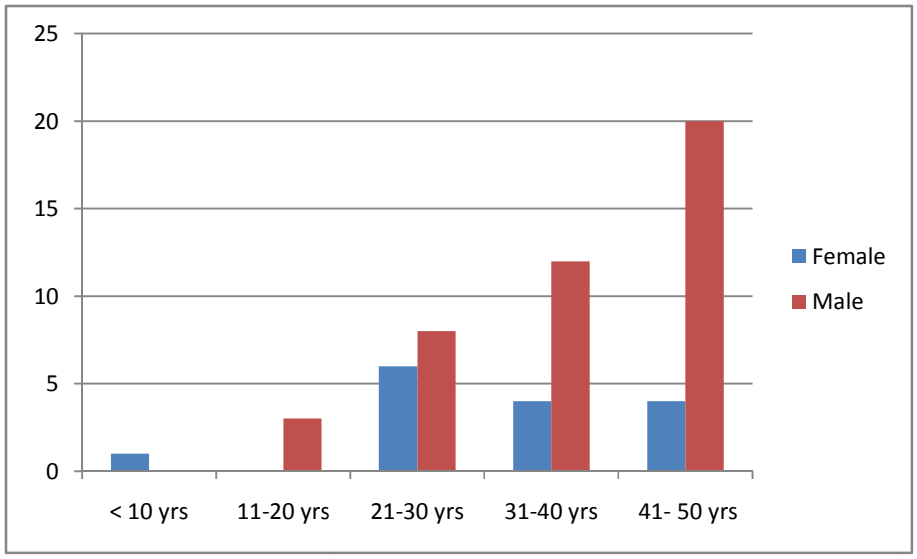
Table18: Susceptibility data of the isolates for four drugs – Griseofulvin, Ketoconazole, Fluconazole, Terbinafine

Drugs		Isolates				
	MIC (µg/ml)	T.mentagrophyte	T.rubrum	Aspergillus	Penicillium	Candida
Griseofulvin	Range	4 - 16	1 – 16	≥ 16	≥ 16	-
	MIC50	8	4	-	-	-
	MIC90	16	16	16	16	-
Ketoconazole	Range	0.06 – 1	2 – 8	-	1 – 16	1 – 8
	MIC50	0.12	4	-	2	2
	MIC90	0.25	8	-	8	8
Fluconazole	Range	0.5 – 8	2 - 16	-	≥ 64	0.25 – 8
	MIC50	2	2	-	-	2
	MIC90	4	8	-	64	4
Terbinafine	Range	0.03 – 0.06	0.03 – 0.12	≤ 0.03	0.03 – 0.06	2 – 16
	MIC50	-	0.06	-	-	2
	MIC90	0.03	0.12	0.03	0.03	16

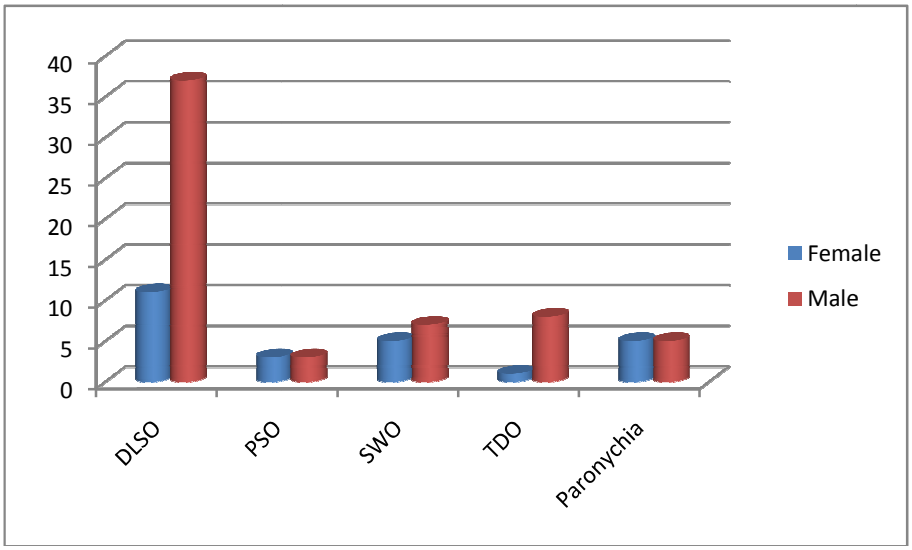
1.SEX WISE DISTRIBUTION OF CASES UNDER STUDY



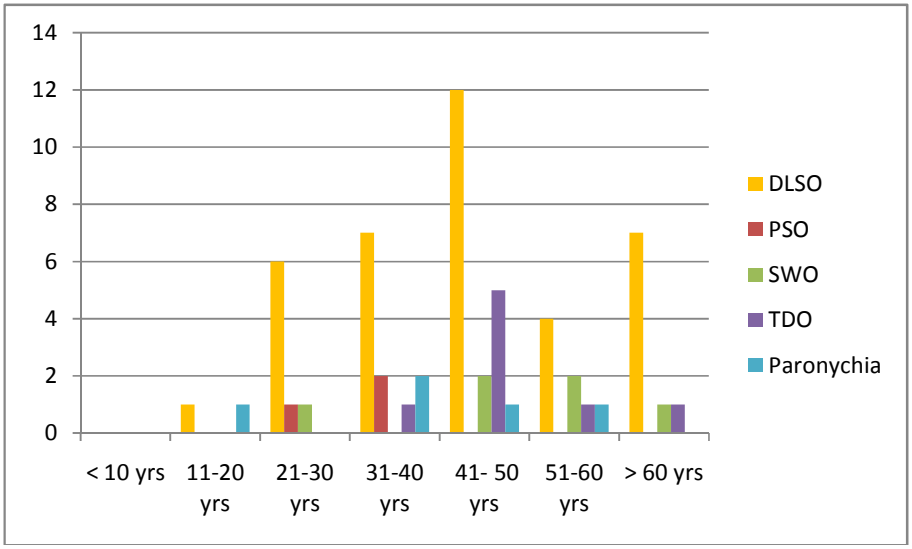
2. AGE AND SEX WISE DISTRIBUTION OF CASES UNDER STUDY



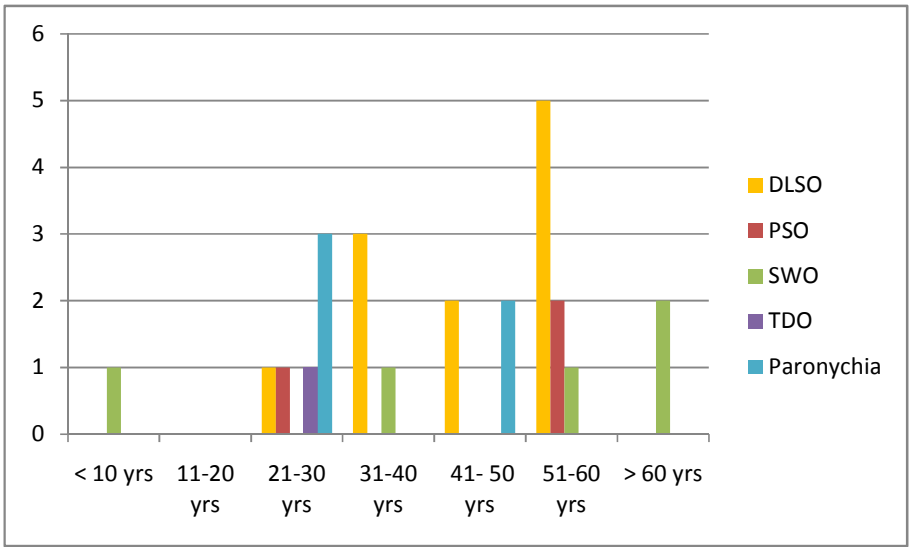
3. SEX WISE DISTRIBUTION OF CLINICAL TYPES



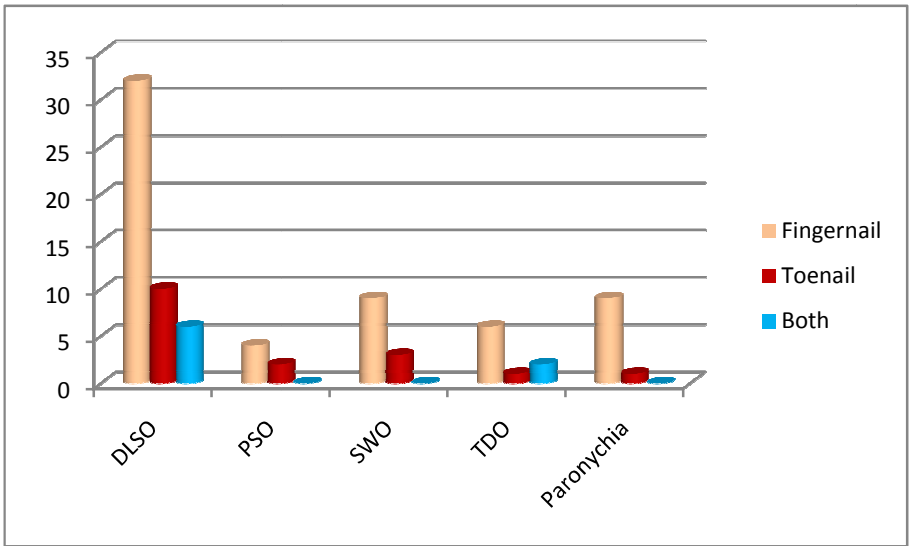
4. AGE WISE DISTRIBUTION OF CLINICAL TYPES AMONG MALES



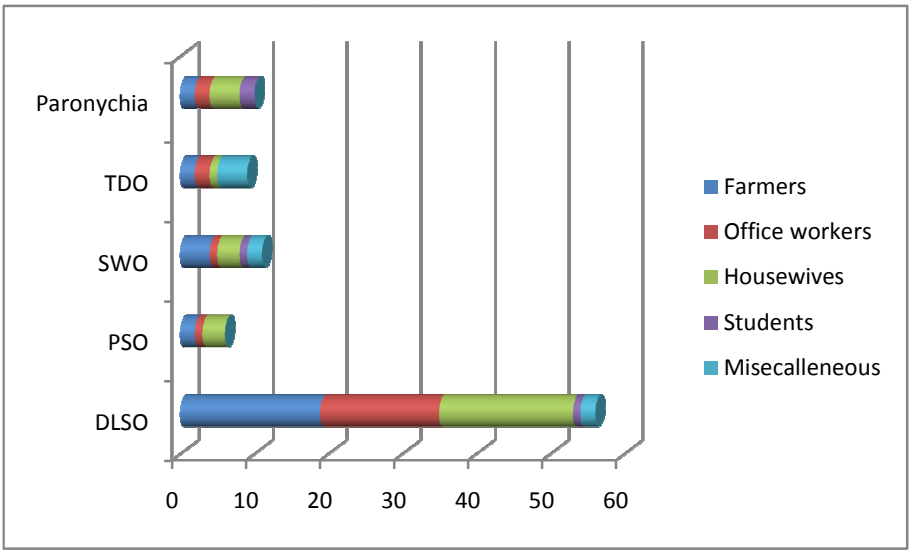
5. AGE WISE DISTRIBUTION OF CLINICAL TYPES AMONG FEMALES



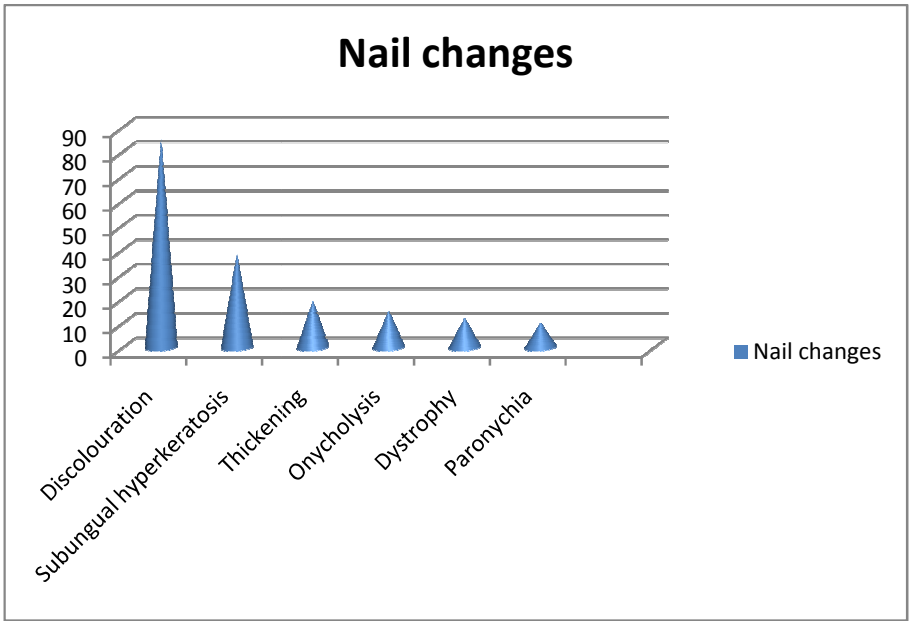
6. CLINICAL TYPES IN RELATION TO SITE OF INVOLVEMENT



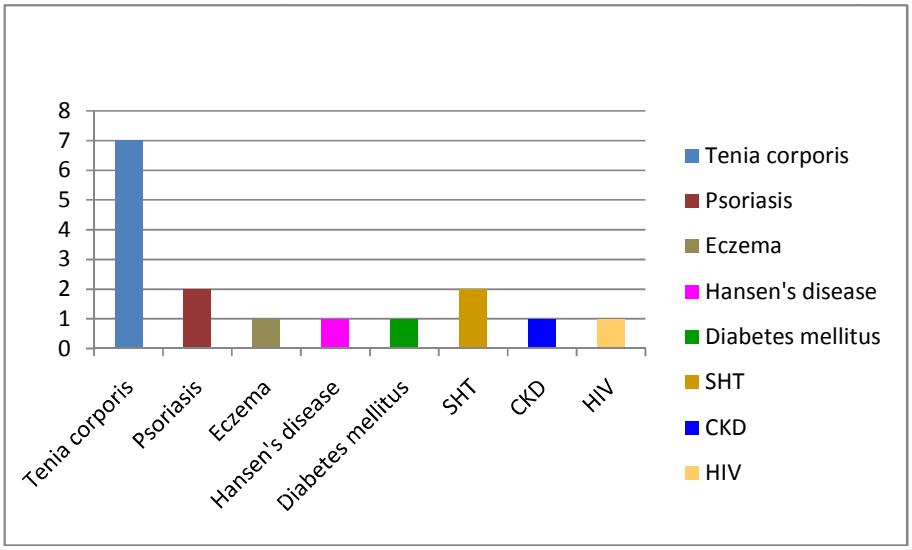
7. CLINICAL TYPES IN RELATION TO OCCUPATION



8. NAIL CHANGES IN ONYCHOMYCOSIS

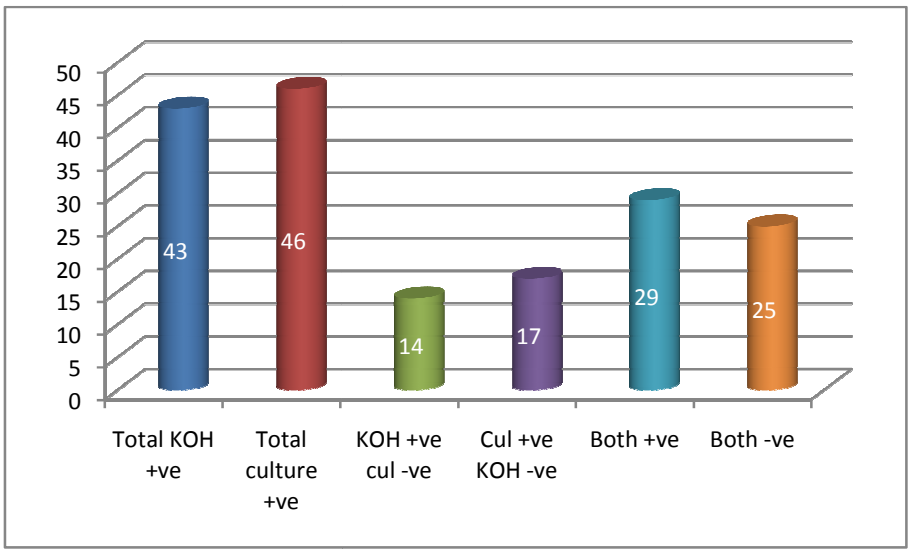


9. ASSOCIATED SKIN & SYSTEMIC DISEASES

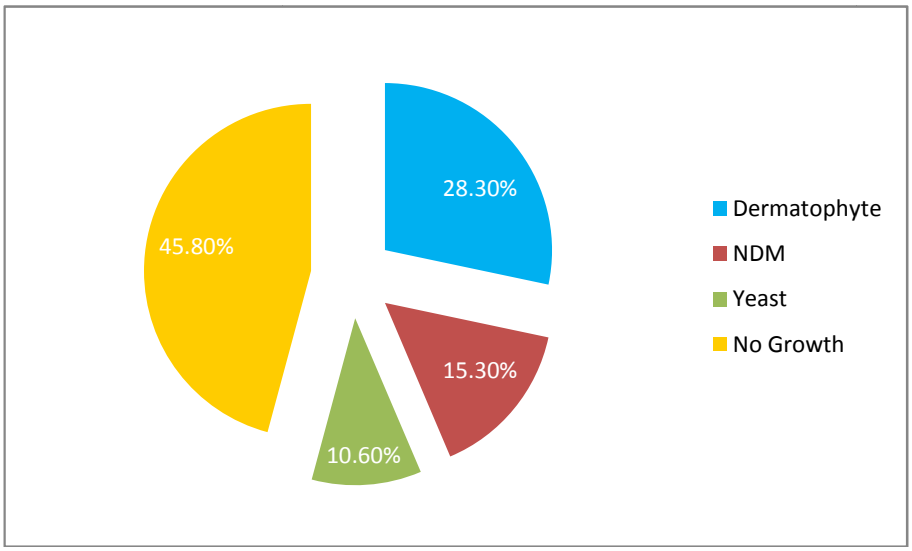


SHT – Systemic Hypertension
CKD – Chronic Kidney Disease
HIV – Human immunodeficiency virus

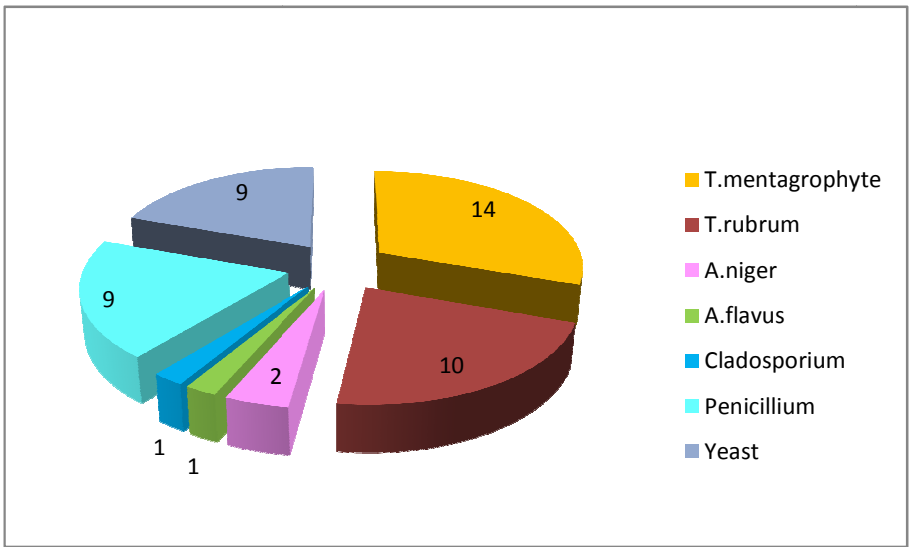
10. MICROSCOPY VS CULTURE RESULTS IN ONYCHUMYCOSIS



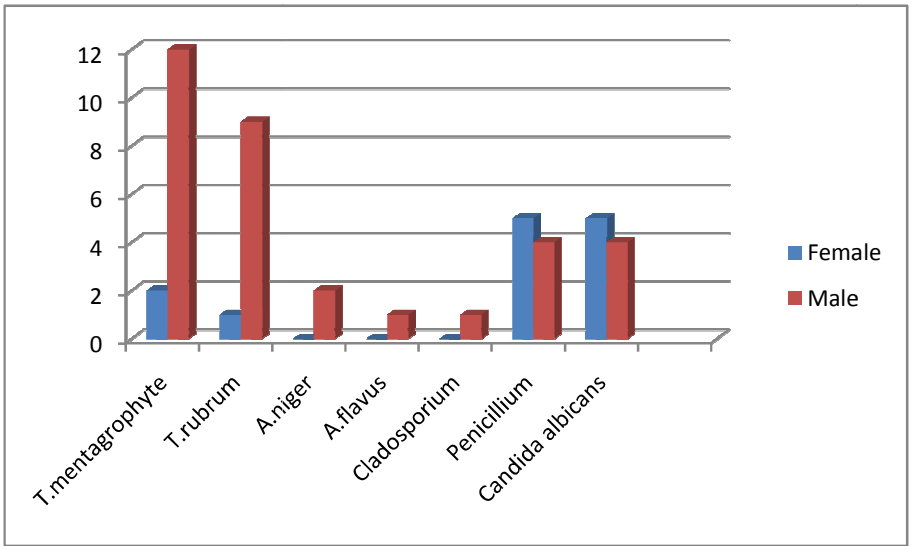
11. PERCENTAGE OF CULTURAL ISOLATES



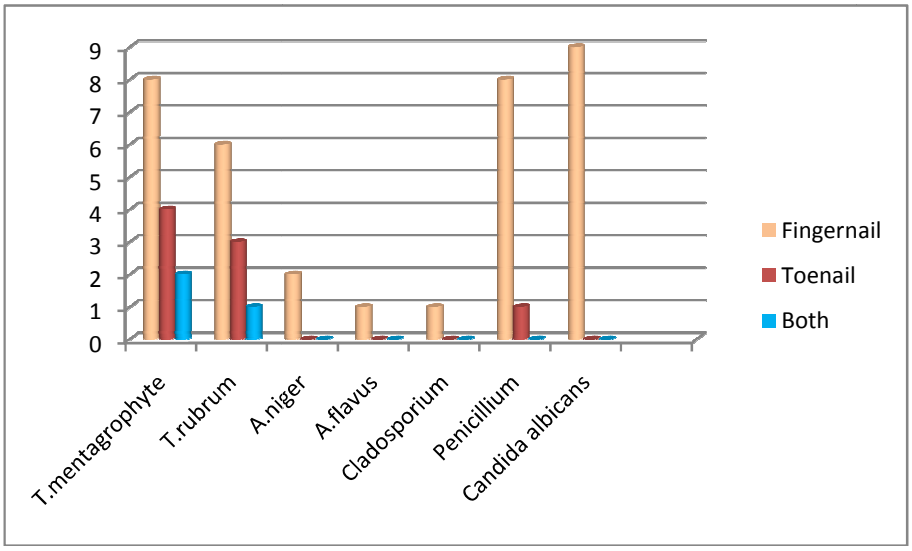
12. DISTRIBUTION OF SPECIES IN ONYCHUMYCOSIS



13. DISTRIBUTION OF SPECIES ACCORDING TO GENDER



14. DISTRIBUTION OF SPECIES ACCORDING TO SITE



DISCUSSION

Onychomycosis is a common infection of nails in adults accounting for the prevalence rate of 2 to 50% worldwide and the incidence increases with age[48]. Various studies from different geographical locations, the incidence of fungal aetiology in clinically pre diagnosed cases of onychomycosis were 43.7% in Poland, 50.6% in Turkey and 43.53% in India.[48, 49,]

In this study, the incidence of onychomycosis was confirmed in 54.12% of clinically diagnosed cases, which is slightly higher than those from more recent Indian studies. Das et al, from Kolkata in 2008 had reported an incidence of 51.76% and Sarma et al from New Delhi had reported even lesser incidence of onychomycosis (32.7%).[15, 21] On the other hand, higher incidence of onychomycosis had also been observed in various countries including India. Pontes et al in Brazil reported 66.5% of confirmed cases and a study by Adhikari et al in 2009 from Sikkim, India showed the incidence of 82.35%.[20, 51]

The slight increase in the incidence of onychomycosis in this study may be attributed to the changing epidemiological factors such as age, social class, occupation, living environment, immuno compromised states etc.

The commonest age group affected in this study was 41 -50 years (28.30%) followed by 31 – 40 years and 51 – 60 years (each constituting 18.80%). So 70% of patients were between the age group of 31 – 60 years which is in accordance with studies [8, 10, 11]by Andre J et al, RSN Brilhante et al and by Gupta et al. The increase in cases with this age group may be justified by repeated nail micro – trauma due to a more prolonged exposure to pathogenic fungi as well as greater work activity and venous insufficiency in this age group.[52, 53] Onychomycosis is uncommon before puberty and the lowest incidence in the present study, was found in <10years of age, 1% which was the same observed in most of the studies.[7, 15, 16,

In the present study men were more commonly affected than women, the ratio being 2.7:1 which compares well with most of the other studies.[17, 19, 22] this may be because men are more exposed to out door works with greater physical activity and are more prone to trauma. However, there have been few studies where men and women are equally affected[21] and even with female predominance.[10]

Various authors and literatures have documented high incidence of onychomycosis in toe nails.[2, 4, 20, 31]In the present study, finger nails were involved more than toe nails with the ratio of 3:1, which was comparable with other studies.[15, 17, 22]Incidence of increased finger nail onychomycosis in this study could be because of the increased chances of occupation related trauma, also finger nail infection makes the patient to seek medical attention than toe nails.

Similar to previous studies, DLSO was the most common clinical type in this study too. The second common clinical presentation was WSO type which is in agreement with Arun Agarwalla et al[54] and Vinod et al[55] studies. Gupta et al[19] had reported the candidal onychomycosis as the second common clinical type. There was one HIV positive patient who had the clinical type, TDO in this study which is similar to Gupta et al study. There was significant association between fingernail involvement and the Paronychia clinical type (P value = 0.007, i.e., < 0.05).

Regarding occupation, the majority of patients in the present study were farmers (29, 34.11%) followed by office workers (22, 25.88%) and house wives (21, 24.71%). Gupta el al[19] from Shimla had reported similar observation in his study. High incidence among these occupational groups is explained by occupation related trauma, increased perspiration and frequent exposure to soil saprophytes. Office workers are educated people who are more

concerned about cosmetic and health consciousness so that they could easily access to health care system. There was significant association between the occupation (farmers) and onychomycosis (P value = 0.01 i.e., <0.05)

Direct microscopy (40%KOH) and the fungal culture together remains the gold standard mycological techniques used in the diagnosis of onychomycosis.[57] Among various studies.[7, 15, 19, 21] microscopic positivity and culture positivity results ranged from 33% - 82% and 37% - 95% respectively. In this study, direct microscopy was positive in 50.59% of cases and the culture was positive in 54.12% of cases which fell within the ranges of other studies.

The two microscopic techniques had almost equal sensitivity of 50.6% (KOH alone) and 48.2% (KOH with DMSO) respectively except the advantage of DMSO in that it gave results in one hour. The P value for both the techniques were greater than 0.05.

The commonest fungi isolated in this study was dermatophytes, 24 (28.3%, n = 85) followed by Non dermatophytic moulds (NDM), 13 (15.3%). Yeast constituted 9 (10.6%) all of which were Candida albicans. Veer et al[17] in 2006 from Aurangabad, India had reported the similar findings with dermatophytes, 29.5%. However, in his study the NDM and Yeasts constituted only 13% and 5.6% respectively. The isolation rate of Yeast and NDM was found to be 5 – 40% and 5 – 35% respectively from various studies.[7, 14, 20, 21] Candidal onychomycosis is commonly associated with finger nail onychomycosis that too in female patients.[14] Here in this study, however there was no significant association between the Candidal onychomycosis and the female patients (P value = 0.085).

Among the species, T.mentagrophyte was the commonest species isolated (14) in this study which is comparable with the Kaur R et al[30], Grover C et al[56] studies from New Delhi and Arun Agarwalla et al[54] study from Nepal. M. Gerami shoar et al[14] from Iran had observed

similar finding in his study. T. rubrum was the second common isolate(10) in the present study. Veer et al[17], Das et al[19] and various other authors from different parts of India[19, 22] had reported T.rubrum as the first common species. However, Adhikari et al[20] from Sikkim had reported T. tonsurans (44.44%) as the commonest species.

In this study there was significant number (9, 10.6%) of Penicillium species among the NDM which is in contrast with most of the other studies[17, 21, 22]where Aspergillus species was the common NDM. Other NDM isolated here were Aspergillus niger (2, 2.4%), Aspergillus flavus (1, 1.2%) and Cladosporium (1, 1.2%). However Adhikari et al from Sikkim had also reported significant number (14.29%) of Penicillium species in his study.

Regarding Candidal onychomycosis, it was commonly found among females in various studies. [7, 54] In this study too female predominance was observed, in whom most of them were house maids. This may be attributed to higher contact time with water in those patients.

In the present study, 16 patients (18.9%) had other associated skin diseases like Tenia Corporis, Eczema, Psoriasis and systemic diseases like SHT, HIV, CRF Among the other dermatological infections Tenia corporis (7, 8.2%) was seen as the commonest one. The association between onychomycosis and the Tenia corporis was just significant (P value = 0.05). So in those patients, the nail infection could serve as the chronic reservoir for the skin infection. S. Neupane et al had recorded 58.2% of onychomycosis was accompanied by superficial fungal infection at other sites, Tenia pedis (25.3%) being the most common infection.

ANTIFUNGAL SUSCEPTIBILITY TESTING (CLSI BROTH MICRODILUTION METHOD M38 A & M27 A2)

Antifungal susceptibility testing of the isolates was done for four drugs namely, Griseofulvin, Ketoconazole, Fluconazole and Terbinafine by using CLSI M38 A and M27 A2 protocols (Espinel Ingroff) for filamentous fungi and yeast respectively. In the present study, the MIC range, MIC50 and MIC90 of Griseofulvin to the dermatophytes was 1 - 16µg/ml, 4µg/ml and 16µg/ml. In contrast, a study by M.E.S. Barros et al[59], 2006 showed the values were 0.062 -1 µg/ml, 0.5 µg/ml and 0.25 µg/ml respectively. A study by Ghannoum et al, 2004[60] showed the MIC range as 0.125 – 64 µg/ml.

For the Aspergillus species and Penicillium species the MIC90 of Griseofulvin was 16µg/ml. Develoux[61] and Artis et al[62] considered isolates resistant to Griseofulvin when MIC values were equal or greater than 3 µg/ml. According to this standard our findings suggest that all the isolates showed in vitro resistance to Griseofulvin. (MIC90 - 16µg/ml).

The MIC range, MIC50 and MIC90 of ketoconazole for dermatophytes were 0.06 – 8 µg/ml, 0.12 µg/ml and 8 µg/ml respectively. These findings correlated with A. K. Gupta and Kohli et al study[63] where the values were 0.06 – 8 µg/ml, 0.125 µg/ml and 2 µg/ml. MIC range, MIC50 and MIC90 for Penicillium species were 1 – 16 µg/ml, 2 µg/ml and 8 µg/ml in this study. For Candida albicans the values were 1 – 8 µg/ml, 2 µg/ml and 8 µg/ml which were close to A. K. Gupta and Kohli et al study[63] where the MIC50 was 1 µg/ml.

The MIC50 and the MIC90 of fluconazole for dermatophytes in this study were 2 µg/ml and 8 µg/ml which varied from other studies, [59, 64, 67] where the MIC50 and MIC90 were high (16 µg/ml and 32 µg/ml). This variation may be influenced by inoculum size for the azole group of drugs.[65] Fluconazole gave highest MIC90 for all Penicillium species in this

study which was 64 µg/ml. Candida albicans MIC range, MIC50 and MIC 90 were 0.25 – 8 µg/ml, 2 µg/ml and 4 µg/ml respectively which was similar to the values observed in Pfaller et al[66]study (MIC90 - ≤8 µg/ml).

Terbinafine showed the lowest MIC value (≤0.03 µg/ml) for all the isolates tested except for Candida albicans to which it showed variable sensitivity (MIC range: 2 -16 µg/ml). These findings compare well with all the other studies.[59, 60, 64]

SUMMARY

- This prospective study was conducted over a period of 15 months and the study population comprised of 85 clinically diagnosed cases of onychomycosis.
- There were 62 cases of Men and 23 cases of Women with a ratio of 2.7:1.
- Most of the cases occurred in the age group range 31 – 60years.
- Lowest incidence of the disease was observed in less than 20years of age group (4.7%).
- The commonest clinical pattern was DLSO (57%) and the less common type was PSO (7%).
- One HIV patient on ART had TDO type of onychomycosis with all nail units affected. The isolate obtained from this patient was T.mentagrophyte.
- Finger nails (70%) were commonly affected than toe nails (20%) with a ratio of 3.5:1.
- The commonly affected occupational group were farmers followed by office workers and house wives. All of them had significant fingernail involvement.
- The associated other skin and systemic diseases were seen in small number of patients (18.9%)
- Direct microscopy was able to detect fungal elements in 43 cases (50.59%) and the culture was positive in 46 cases (54.12%)
- Group of fungi involved in these cases were Dermatophytes, Non Dermatophytic moulds and the Yeast.
- T.mentagrophyte was the commonest species among dermatophytes and Penicillium species was the common isolate among NDM, while all the Yeasts were Candida albicans.
- Anti fungal susceptibility testing of the isolates was done by CLSI broth micro dilution method (M38 A & M27 A2).

- MIC50, MIC90 was obtained for four drugs namely, Griseofulvin, Ketoconazole, Fluconazole and Terbinafine.
- All the isolates were resistant to Griseofulvin with the MIC 16 µg/ml.
- All the isolates were sensitive to Terbinafine, Ketoconazole, Fluconazole with the exception of Penicillium species showing resistant to Fluconazole.
- Among all the drugs tested, Terbinafine showed the lowest MIC, ≤0.03 µg/ml for all the isolates except for Candida albicans.
- For all the filamentous fungi, Terbinafine was the best drug with highest in vitro activity.
- Fluconazole was the better choice for Candida albicans.

CONCLUSION

The findings in this study revealed that onychomycosis is an important health problem in Coimbatore district, South India and the epidemiological pattern of the disease varied from other parts of the country. The same clinical type of disease yielded different fungal species in culture so that type of nail changes cannot be taken as a reliable marker for predicting the causative agent and thus the study stresses the need for microbiological confirmation in case of onychomycosis.

The sensitivity of direct microscopy and culture together for onychomycosis in this study was 70.58% when compared to Microscopy (51%) or culture (54%) alone. So this study concludes that direct microscopy should always be coupled with fungal culture for the accurate diagnosis and species identification to carry out the appropriate treatment for this chronic relapsing disease.

The CLSI broth microdilution method (M38 A & M27 A2) was used for in vitro susceptibility testing of the isolates because of its reproducibility, reliability and user friendly nature. The in vitro data obtained in this study indicates that the terbinafine is the most potent of all antifungal agents with lowest MIC to treat all filamentous fungi, both the dermatophytes and the non dermatophytic moulds causing onychomycosis which is further confirmed by other studies, [39] the relatively high clinical cure (in vivo activity) rate of 80-90% for finger nail infections and 70 – 80% for toe nail infections.

The results also suggest that the best drug for treating Candidal onychomycosis is fluconazole. The griseofulvin, which has been extensively used to treat superficial fungal infections including onychomycosis in our clinical setting showed the highest MIC (≥16μg/ml) in the present study which correlated well with the poor clinical outcome. This study also stresses that the Penicillium species is the significant NDM causing onychomycosis in this area and all the Penicillium isolates showed in vitro resistance to fluconazole (MIC ≥64μg/ml) in this study.

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LIST OF ABBRIVIATIONS

DLSO	Distal lateral subungual onychomycosis
PSO	Proximal subungual onychomycosis
SWO	Superficial white onychomycosis
TDO	Total dystrophic onychomycosis
NDM	Non dermatophytic mould
T.mentagrophyte	Trichophyton mentagrophyte
T.rubrum	Trichophyton rubrum
spp	species
DMSO	Dimethyl sulphoxide
RPMI	Rose Parkers Memorial Institute
MIC	Minimal Inhibitory Concentration
CLSI	Clinical Laboratory Standard Institute
SHT	Systemic Hypertension
CKD	Chronic Kidney Disease
CRF	Chronic Renal Failure
HIV	Human Immuno Deficiency Virus

APPENDIX

1) PREPARATION OF 40% KOH:

Potassium hydroxide - 40 gm

Distilled water - 100 ml

Add Potassium hydroxide crystals to distilled water slowly and with stirring mix by stirring until the crystals are dissolved.

2) PREPARATION OF 40% KOH with DMSO:

Potassium hydroxide - 40 gm

Dimethyl sulphoxide – 40ml

Distilled water - 60 ml

Add Potassium hydroxide crystals to distilled water slowly and with stirring mix by stirring until the crystals are dissolved. Then add 40ml 0f DMSO in to that solution and mix well.

3. LACTOPHENOL COTTON BLUE:

Phenol - 20 gm

Lactic acid - 20 ml

Glycerol - 40 ml

Distilled water - 20 ml

Cotton blue - 0.075 gm

Add Lactic acid and glycerol to distilled water. Mix well. Add phenol crystals and dissolve by gentle heating. Add cotton blue and dissolve thoroughly.

The phenol acts as disinfectant, Lactic acid preserves the morphology of the fungi and glycerol is hygroscopic agent which prevents drying. The cotton blue stains the outer wall of the fungus.

4. SABOURAUD'S DEXTROSE AGAR:

Dextrose - 40 g

Peptone (mycological) - 10 g

Agar - 20 g

Distilled water - 1000 ml

(65 grams of dehydrated media should be reconstituted in 1 litre of distilled water).

Dissolve the powder in distilled water by boiling. Adjust the pH to 5.4. Autoclave at 121°C for 15 minutes at 15 lbs. Dispense in tubes and in Petri dishes. Allow the tubes to cool in slanting position. Store it in refrigerator.

5. SABOURAUD'S DEXTROSE AGAR WITH CHLORAMPHENICOL AND CYCLOHEXIMIDE:

Dextrose - 40 g

Peptone (mycological) - 10 g

Agar – 20 g

Chloramphenicol – 50 mg

Cycloheximide – 500 mg

Prepare Sabouraud's dextrose agar by dissolving the ingredients in distilled water as explained above. Dissolve 50 mg of chloramphenicol in 10 ml of 95% alcohol and 500 mg of cycloheximide in 10 ml of acetone and add to the boiling medium. Remove from heating and mix well. Dispense in tubes and autoclave at 121°C for 15 minutes.

6. POTATO DEXTROSE AGAR:

Potato: 200g

Dextrose: 20g

Agar: 20g

Water: 1 lit

Scrub but do not peel the potato & cut into 12 ml cubes, Boil 200g in 1 lit of water for 60 minutes. Squeeze as much of the pulp as possible through a fine sieve. Add agar and boiled till dissolved. Add dextrose and make up to 1 liter. Dispense in required amounts taking care to keep solids in suspension. Autoclave at 115° C for 30 minutes. Cool to 50° C & pour in to tubes and allowed to cool in slanted position.

7. CHRISTENSEN'S UREA MEDIUM:

Peptone: 1.0g

NaCl: 5.0g

KH2 PO4: 2.0g

Glucose: 5.0g

Agar: 20.0g

Distilled water: 1000ml

After dissolving the above ingredients by heat 5ml of phenol red solution (0.2% in 50 % alcohol) was added after which autoclaved at 115° centigrade per 15 minutes. On cooling 100ml of urea (20% aqueous solution sterilized by filtration) is added medium is poured into slopes.

PROFORMA

NAME: AGE/SEX: OP NO:

ADDRESS/LOCALITY: DATE:

SOCIO-ECONOMIC STATUS:

Educational level:
Occupation :
Living condition :
Others :

PERSONAL HISTORY:

PAST HISTORY:

TREATMENT HISTORY, IF ANY: (**In the past)**

PRESENTING COMPLAINTS (WITH DURATION):

FIRST EPISODE:

LOCAL EXAMINATION OF INFECTED SITE:(**NAIL**)

a) FINGER NAIL/TOE NAIL

b) SITE :

i) Colour : ii)Texture : iii)Nail plate : iv) Nail bed :

v) Subungual hyperkeratosis : vi) Onycholysis :

CLINICAL DIAGNOSIS :

TREATMENT:

FOLLOW UP: Completely resolved/Partially resolved/Not resolved

WORK SHEET

1) CLINICAL SAMPLE: Nail clippings/ scrapings

2) KOH WET MOUNT:
i)YEAST CELLS:
ii) HYPHAE : Septate, Branched hyphae- Mild/moderate/more
Spores, if any
Pigmentation, if any

3) CULTURAL ISOLATION (IN DUPLICATES) AT 25-30ºC

3a) SDA WITH ANTIBIOTICS & ANTIFUNGALS
(Cycloheximide + Chloramphenicol)
Growth characteristics:

1 wk	2 wks	3 wks	4 wks

AT 25-30ºC (NDM)

3b) SDA WITH ANTIBIOTIC (NDM)
Growth characteristics:

1 wk	2 wks	3 wks	4 wks

3c) SDA WITH ANTIBIOTIC (CANDIDA SPP) at 37ºC, 24-48hrs:
Growth characteristics:

4) SPECIES IDENTIFICATION: LPCB mount, Special tests (Urease test, Slide culture, etc)
Candida spp – Grams stain, Germ tube test

MYCOLOGICAL DIAGNOSIS:

5) ANTI FUNGAL SUSCEPTIBILITY TESTING (CLSI M-38 A2 & M27 A2)

Detection of MIC – Microbroth dilution Technique

	MIC µg/ml	Interpretation(R/I/S)
Griseofulvin		
Ketaconazole		
Fluconazole		
Terbinafine		

MASTER CHART

S.No	NAME	OP NO	AGE/ SEX	OCCUPATION	CLINICAL DIAGNOSIS		MYCOLOGICAL DIAGNOSIS		
					Type	Site	40%KOH	40%KOH &DMSO	Culture
1	Thirumoorthi	425867	37/M	O	C/c paronychia	FN Rt	-	-	Candida
2	Sivasamy	110113	42/M	F	DLSO	TN B/L	+	+	-
3	Gurunathan	12605	42/M	F	DLSO	FN B/L	+	-	T.rubrum
4	Dhivakaran	80028	63/M	F	DLSO	TN Rt	+	+	T.rubrum
5	Devaraj	117878	30/M	F	DLSO/ T.corporis	TN Rt	+	+	T.mentagrophyte
6	Thirupathi	163078	30/M	O	DLSO/ T.corporis	TN Lt	+	-	-
7	Balamani	123585	58/F	H	PSO	FN Rt	-	-	Candida
8	Subbaiya	170622	62/M	F	.DLSO/ T.corporis	FN B/L	+	+	-
9	Thulasimani	300904	22/F	H	DLSO	TN Rt	-	-	-
10	Kumar	302658	29/M	O	PSO	FN Rt	-	-	A.niger
11	Ramaraj	400987	60/M	F	C/c paronychia	TN B/L	-	-	Candida
12	Nallasamy	6435/09	65/M	M	DLSO	FN B/L	+	+	A.flavus
13	Subbaiya	470572	65/M	F	DLSO	FN B/L	-	-	T.mentagrophyte
14	Pattiyappan	180253	65/M	F	C/c paronychia	FN Rt	+	-	Candida
15	Prakasam	502617	30/M	O	DLSO	FN Rt	-	-	-

16	Iyyammal	515232	52/F	H	DLSO	FN & TN B/L	+	+	T.rubrum
17	V.Ramani	515278	43/M	O	SWO	TN Rt	+	+	-
18	Nataraj	256524	65/M	O	DLSO	FN Rt	+	+	-
19	Nagalingam	206718	40/M	M	DLSO	FN Lt	+	+	T.mentagrophyte
20	Mallika	528627	30/F	H	TDO/Onycholysis	FN/B/L	+	+	Penicillium
21	Murugasen	54290	39/M	O	DLSO	FN Rt	+	+	T.rubrum
22	Kalaiyarasi	54382	6/Fch	M	SWO	FN Lt	+	+	-
23	Sadikbasha	568796	45/M	M	TDO/Onycholysis	FN B/L	+	+	Penicillium
24	Thirumathal	14790	55/F	H	DLSO	FN/B/L	-	-	T.mentagrophyte
25	Ushainbeevi	435233	58/F	H	DLSO	FN Rt	+	+	Penicillium
26	Sivasamy	549253	47/M	M	TDO/Hansen's dis	FN B/L	+	+	T.mentagrophyte
27	Lakshmi	586907	30/F	H	C/c paronychia	FN Rt	-	-	-
28	Palanisamy	586958	43/M	O	DLSO	FN Rt	+	+	-
29	Kumar	629523	18/M	S	SWO	FN Rt	-	-	-
30	Kannan	629657	52/M	O	DLSO	FN B/L	+	+	-
31	Rangaraj	557561	46/M	F	DLSO	FN Rt	-	-	-
32	Susila	5697	60/F	H	DLSO	TN B/L	-	-	T.mentagrophyte
33	Subbaiya	69007	60/M	F	DLSO/Eczema of hands & legs	FN & TN B/L	+	+	-

34	Rajamani	1698	50/F	H	C/c paronychia	FN Rt	-	-	Candida
35	Shanmugam	205410	45/M	F	DLSO/Psoriasis/DM	FN B/L	-	-	T.rubrum
36	Ravi kumar	21199	26/M	M	SWO/CKD/CRF	FN B/L	+	+	Cladosporium
37	Balraj	700491	40/M	F	DLSO	FN B/L	-	-	-
38	A.Nataraj	710139	40/M	O	TDO	TN Rt	-	-	T.mentagrophyte
39	Raman	711453	50/M	F	DLSO	FN Lt	+	+	-
40	Selvi	725441	30/F	H	PSO	FN Rt	+	+	-
41	Gopal	734824	50/M	F	DLSO	FN B/L	+	+	T.mentagrophyte
42	Palanisamy	734666	40/M	O	DLSO/ T.corporis	FN Rt	-	-	-
43	Ganesh	734325	30/M	O	DLSO	FN Lt	-	-	-
44	Venkatachala	736571	50/M	O	C/c paronychia	FN B/L	-	-	Candida
45	Kunjammal	749690	45/F	H	DLSO	FN B/L	-	-	-
46	Mery	720420	54/F	H	DLSO	FN B/L	+	+	Penicillium
47	Mohammad	720414	52/M	M	TDO/onycholysis	FN B/L	-	-	T.rubrum
48	Mariyappan	8990	35/M	F	PSO/onycholysis	FN Rt	+	+	Candida
49	Uthramoorthy	769832	33/M	O	DLSO	FN Lt	+	+	T.mentagrophyte
50	Shanmugam	769910	65/M	F	DLSO	FN Rt	-	-	-
51	Palanisamy	769928	45/M	O	DLSO	FN Rt	-	-	T.rubrum

52	Rangasamy	776514	65/M	F	DLSO	FN Lt	+	+	-
53	Karupusamy	779400	60/M	F	SWO	FN Lt	+	+	A.niger
54	Ramesh	780188	19/M	S	DLSO	TN Rt	-	-	-
55	Papathi	79522	65/F	F	SWO/SHT/RA	TN B/L	-	-	-
56	Arumugam	506309	60/M	F	DLSO/T.corporis	TN B/L	+	+	T.rubrum
57	Raguram	79540	50/M	F	DLSO	FN Lt	-	-	-
58	Kannammal	541/10	65/F	H	SWO	FN Rt	+	+	Penicillium
59	Kokila	575644	48/F	H	DLSO	FN B/L	-	-	-
60	Amudhavalli	57341	52/F	H	SWO	FN Rt	+	+	Penicillium
61	Rathinum	201234	57/F	H	PSO	FN B/L	-	-	Candida
62	Raheem	34212	30/M	O	DLSO/ T.corporis	TN Rt	-	-	T.mentagro phyte
63	Sulochana	58356	47/F	H	Paronychia	FN Rt	-	-	Candida
64	Sangeetha	81589	21/F	S	Paronychia	FN B/L	+	+	Candida
65	Arulsamy	65910	64/M	F	SWO	TN Rt	+	+	Penicillium
66	Kamatchi	82483	39/F	H	DLSO	FN Rt	-	-	-
67	Rajendran	52604	46/M	M	SWO/SHT/Psoriasis	FN B/L	-	-	-
68	Aelumalai	58666	27/M	F	DLSO	FN Rt	+	+	T.mentagro phyte
69	Nataraj	78077	65/M	F	TDO	FN B/L	-	-	-

70	Shanthamani	735776	38/F	H	DLSO	FN B/L	-	-	-
71	Palanisamy	-	36/M	F	PSO	TN B/L	+	+	-
72	Josaphwilson	-	42/M	O	TDO	FN & TN B/L	+	+	T.mentagrophyte
73	Maragatham	14660	35/F	H	SWO	FN Lt	-	-	-
74	Senthilkumar	51381	42/M	O	DLSO	TN B/L	+	+	-
75	Riyastha	743841	26/F	H	Paronychia	FN Rt	-	-	-
76	Thangavel	7853	56/M	F	DLSO	TN B/L	-	-	T.rubrum
77	Chenniyappan	18401	45/M	F	TDO	FN Rt	+	+	T.mentagrophyte
78	Kandhasamy	167352	60/M	F	SWO	FN Lt	-	-	-
79	Kaalisamy	642042	55/M	F	DLSO	FN Rt	-	-	-
80	Ganesh	17500	37/M	O	DLSO/Tenia corporis	FN B/L	-	-	T.rubrum
81	John wilson	1862/10	45/M	M	AIDS/TDO	FN & TN B/L	+	+	T.mentagrophyte
82	Balakumar	1802	19/M	S	Paronychia	FN B/L	+	+	Penicillium
83	Dhakshinamoorthy	18659	45/M	F	DLSO	FN & TN B/L	-	-	-
84	Udhayakumar	154551	35/M	O	DLSO	FN Rt	+	+	Penicillium
85	Sivakumar	-	37/M	O	DLSO	FN Rt	-	-	-

KEY TO MASTER CHART

Sex:

M – Male

F - Female

Occupation:

F – Farmer

O – Office worker

H – House wife

S – Student

M – Miscellaneous

Site:

FN – Fingernail

TN – Toe nail

Rt - Right side

Lt – Left side

B/L - Bilateral